MICROBIAL DEGRADATION OF CHLOROPHENOLS IN BATCH AND CONTINUOUS BIOREACTORS: KINETIC STUDY AND OPTIMIZATION OF PROCESS PARAMETERS

A thesis submitted in partial fulfillment for the award of the degree of

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Submitted by

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CERTIFICATE

This is to certify that the thesis entitled "***Microbial Degradation of Chlorophenols in Batch and Continuous Bioreactors: Kinetic Study and Optimization of Process Parameters***" by Mr. Bhishma Patel submitted to the **National Institute of Technology**, **Rourkela** for the Degree of ***Doctor of Philosophy*** in ***Chemical Engineering***, is a record of bonafide research work, carried out by him under my supervision and guidance. I believe that the thesis fulfils part of the requirements for the award of ***Doctor of Philosophy***. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/Institute for the award of any Degree or Diploma.

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Rourkela **Bhishma Patel**

Date:

Dedicated to

Aekta

Rushika

Vivaan

and

My Parents

ABSTRACT

Chlorophenols are known to be detrimental to the environment owing to their physiochemical properties, which result in a higher persistence and subsequent bioaccumulation. Chlorophenols have been reported for their carcinogenicity, immunogenicity, mutagenicity and fatality to humans and other organisms. Biodegradation is an eco-friendly, energy efficient and potential alternative to various physicochemical treatment methods used for environmental pollution abatement with promising removal efficiency. The main objective of the study is to propose a scheme for efficient treatment of chlorophenol containing wastewater. Effective implementation of biological methods have limitations and requires the understanding of the complex interaction between substrate and microorganisms. Wastewaters, both domestic as well as industrial, commonly encounter a mixture of different recalcitrant synthetic organic compounds (SOC) along with biogenic substrates. The common treatment plants are unable to treat recalcitrant SOC effectively and they generally pass through without complete treatment. Hence a complete understanding of the biological methods and various other parameters involved is highly necessary for achieving the anticipated results.

In the first part of the study, eight different bacterial strains were isolated from a two different possible sources which has high chance of chlorophenol contamination. Out of these eight strains, four pure strains were selected for further study based on their high tolerance and degradation capacity for chlorophenols. The isolated strains were characterized morphologically, biochemically as well as genetically and they were identified as *Bacillus cereus*, *Bacillus endophyticus*, *Kocuria rhizophila* and *Pseudomonas aeruginosa*. The 16S rDNA sequences have been indexed in GenBank database for public acknowledgement. Process optimization could enhance the treatment of recalcitrant compounds efficiently. In the second part of the research, pure strains were tested for degradation of six different chlorophenols (2-CP, 3-CP, 4-CP, 2,4-DCP, 2,4,6-TCP, and PCP). Using Response Surface Methodology, four experimental parameters were optimized and proven to be effective to achieve maximum degradation and tolerance for chlorophenols. The biodegradation kinetics and growth kinetics of the strains were also evaluated. The ability of the microbes to co-metabolize 2,4-DCP and mono-chlorophenol has also been assessed in this part. Next, the biodegradation of chlorophenols and its co-metabolism (multi-substrate degradation) by the two different mixed microbial consortia, one defined consortium and the other one undefined consortium was carried out. The biodegradation kinetics was studied using Andrews's substrate inhibition model for biodegradation of chlorophenols by the mixed consortium. The final part of the study focused on the continuous degradation of chlorophenols in three customized bioreactors: two packed bed biofilm reactor (PBBR) and one airlift inner loop reactor (ALR). In the first packed bed biofilm reactor (PBBR-1), the biodegradation of 2,4-dichlorphenol by two different isolated strains was studied. In the second packed bed biofilm reactor (PBBR-2), the biodegradation of 3-chlorophenol and 4-chlorophenol by the defined mixed microbial consortium was studied individually. In the third bioreactor i.e., airlift inner

loop reactor (ALR), two different studies have been conducted. First, the biodegradation of 4-chlorophenols by the mixed microbial consortium (undefined) was explained with the metabolic pathway. Second, the biodegradation of mixture of different chlorophenols including 2-chlorophenol, 3-chlorpohenol, 4-chlorophenol, 2,4-dichlorophenol, and 2,4,6-trichlorophenol was conducted. In all the three bioreactor studies, the effect of hydraulic retention time, initial substrate concentration or loading rate and effect of biogenic substrate concentration on removal efficiency of chlorophenols in bioreactors during continuous mode were evaluated extensively.

From the experimental results obtained, the general hypothesis considered for the present work were been evaluated. The results indicated that, acclimatization of a microbe to a certain compound has a profound effect on its tolerance towards the compound. The pure strains were able to degrade only 2-CP and 2,4-DCP but unable to degrade 3-CP, 4-CP, 2,4,6-TCP and PCP. Next, the biodegradation results obtained by mixed microbial consortia makes them a potential option not only for complete mineralization of the recalcitrant compounds but also for their ability to treat various compounds simultaneously. Among the two different consortia studied, the defined mixed consortium showed excellent prospective for degradation of lower to higher chlorophenols in single and mixture as compared to the undefined one. The biodegradation kinetics of chlorophenols gives an important insight for comparison of the degradation process and can help in designing and implementing a bioreactor.

Finally, the bioreactor studies and effect of various parameters proven very effective for understanding the degradation of chlorophenols and its complete removal. The effect of biogenic substrate showed that, the presence of higher concentration of biogenic substrate do not affect the removal efficiency of chlorophenols in bioreactors except 3-CP. As a matter of fact, at lower biogenic substrate concentration, the removal efficiency and biodegradation rate increased for chlorophenols. The effect of hydraulic retention time and loading rate indicated that, there are two different optimized conditions that can be applied based on the selected outcome i.e. maximum volumetric removal and maximum biodegradation of the chlorophenols.

This study is important as it will provide the information which has direct relevance towards the applicability of microbes for degradation of organic pollutant in the environment. Knowledge acquired from the biodegradation processes and their mechanisms for degradation of chloro-aromatic compounds provides a better understanding of fate and cycling of chlorophenolic compounds in the environment.

Keywords: Chlorophenols; Biodegradation; Kinetics; Bioreactor; Biofilm; Airlift reactor; Packed bed reactor; Optimization; Response surface methodology

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ABBREVIATIONS

Rs	Actual rate of dergadation
Rm	Maximum rate of degradation
μ	Specific growth rate
μ_m	Maximum specific growth rate
K_s	Half saturation constant
K_i	Substrate inhibition constant
S_{max} or S^*	Critical substrate concentration
CHMS	5-chloro-2-hydroxymuconic semialdehyde
MCP	Monochlorophenol
DCP	Dichlorophenol
TCP	Trichlorophenol
2-CP	2-chlorophenol
3-CP	3-chlorophenol
4-CP	4-chlorophenol
2,4-DCP	2,4-dichlorophenol
2,4,6-TCP	2,4,6-trichlorophenol
TeCP	Tetrachlorophenol
PCP	Pentachlorophenol
CC	Chlorocatechol
MSM	Mineral salt medium
RSM	Response Surface Methodology
CCD	Central Composite Design
ANOVA	Analysis of Variance
HPLC	High Pressure Liquid Chromatography
MS	Mass Spectroscopy
FESEM	Field Emission Scanning Electron Microscope
IARC	International Agency for Research on Cancers
PBBR	Packed bed biofilm reactor
ALR	Airlift inner loop reactor
PBR	Packed bed reactor
FBR	Fluidized bed reactor
UASB	Upflow anaerobic sludge blanket

HRT	Hydraulic Retention Time
NCBI	National Center for Biotechnology Information
BLAST	Basic local alignment tool
PCR	Polymerase chain reaction
RDP	Ribosomal database project
MEGA-5	Molecular evolutionary genetics analysis software v.5
ATSDR	Agency for toxic substances and disease registry
EPA	Environmental protection agency
EDTA	Ethylenediaminetetraacetic Acid
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
OD	Optical density
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
VSS	Volatile Suspended Solids
ppm	Parts per Million
ppb	Parts per Billion
RPM	Rotation per Minute

CHAPTER – I

INTRODUCTION

CHAPTER - I

INTRODUCTION

1. GENERAL

With the advent of heavy industrialization, activities polluting the environment and concomitantly sabotaging the inherent ecosystems have enormously increased. Tremendous amounts of toxic, hazardous pollutants have been generatedin the environment through industrial, agricultural, medical, and domestic activities. Most of these contaminants are xenobiotic and recalcitrant in nature, which makes them highly persistent and bioaccumulative in the environment. Chlorophenols constitute a group of one of these contaminants, which have been classified as priority pollutants by the US Environmental Protection Agency [1]. They are regarded as a serious menace to the environment because of their widespread occurrence in the soil, sediments, sludge products, wastewater, surface water, and groundwater [2, 3].

Chlorophenols contain 19 different congeners including mono-, di-, tri-, tetra-, and pentachlorophenol, among these eight chlorophenols are commercially used. Recent reports indicate that presence of chlorophenols in environment imposes several health impacts on humans and the surrounding natural biodiversity. Chlorophenol compounds may cause some histopathological alteration, mutagenicity genotoxicity, carcinogenicity and immunogenicity in humans and other organisms [4, 5]. The International Agency for Research on Cancers (IARC) have classified 2,4,6-TCP, 2,4,5-TCP and Pentachlorophenol as group 2B possible carcinogen to human [6, 7]. Chlorophenols interfere with the metabolizing enzymes that lead to increased respiration rate [8]. Daniel et al. (2001) manifested the possible relationship between blood levels of PCP and immune function. It's been concluded that increased blood levels of PCP were associated significantly with cellular and humoral immune deficiencies. Also, the patients suffered from frequent respiratory infections and general fatigue [9]. Therefore, their fate into the environment is of great concern.

Various physicochemical treatments such as adsorption, photo degradation, chemical oxidation, solvent extraction, etc. have been developed for treating chlorophenols contaminated soil and water [10]. Although chemical treatments are faster than biological treatment and also can treat high strength and loading of toxic compounds, the cost of these treatments is prohibitive for large-scale application, are low energy efficient and some treatments need secondary treatment due to production of toxic byproducts [11]. These treatment processes convert or transforms the toxic compounds from one phase to another phase only, which subsequently requires further processing for complete mineralization.

However, biological treatment technologies such as the use of microorganism and enzymes have been shown to be a potential alternative with promising removal efficiency [3, 12]. In the recent past years, the use of biological treatment technology for removal of toxic compounds increased because of its higher energy efficiency and complete mineralization of

the toxic compounds. Both aerobic and anaerobic biodegradation processes are important in the expected overall treatment schemes. However, effective implementation of biological methods has limitation and requires the understanding of the complex interaction between substrate and microorganisms. Wastewater treatment plant commonly contains the mixture of different recalcitrant synthetic organic compounds (SOC) along with biogenic substrates. The common treatment plants are not able to treat recalcitrant SOC effectively and passes through without complete treatment [13]. Process optimization can improve the treatment of recalcitrant compounds. These require the understanding of biomass acclimation, interaction between the biogenic substrate and recalcitrant compounds, reactor operating conditions for SOC treatment, biofilm and biodegradation kinetics.

1.1 Motivation and hypothesis

It has been reported that acclimation of the microorganisms to recalcitrant compounds or specific SOC can substantially improve the degradation process over time. However, it was not clear that the acclimation of the microbial community to one specific recalcitrant compounds can effectively utilize other recalcitrant compounds. The specific group of microbes or competent biomass present in the microbial community are responsible for the removal of specific SOCs [14]. During the acclimation of the microbial community, it has been observed that the competent biomass or specific microbes requires for degradation of specific SOCs are increases in the medium. Also, the isolation source of the microbes has an effect on the biodegradation of specific SOCs. Goel et al., (2009) reported the effect of inoculum source on biodegradation of chlorophenols [15].

Another important factor that affects the biodegradation of chlorophenols is the presence of biogenic substrate. As mentioned previously that, the specific competent biomass is responsible for thedegradation of the specific compound, which is equal to the fraction of COD contributed to the feed by that compound [13, 16]. So, the presence of biogenic substrate does not guarantee the improved biodegradation of recalcitrant compounds. These phenomena also reported in the literature. Hu et al., (2005) reportedthat the addition of biogenic substrate decrease the degradation rate of 4-chlorophenol, whereas it enhance the degradation of 2,4-dichlorophenol. Sahinkaya and Dilek (2006) studied the effect of biogenic substrate on the removal of 4-chloropohenol and 2,4-dichlorophenol mixture in the sequencing batch reactor. They found that presence of biogenic substrate helps in complete removal of chlorophenol mixture, and also it helps to overcome the competitive inhibition of both compounds in the reactor [17].

Co-metabolism of chlorophenols is important for understanding the practical application of microorganisms for bioremediation and synergetic of the biodegradation. Also, it helps in understanding the multi-substrate degradation process thathas been encountered in the *in-situ* environment. In the environment, more than one chlorophenol congeners are present together along with other aromatic compounds. Interaction of these recalcitrant compounds is complex and has a great influence on the biodegradation of chlorophenols due to toxicity, competition, molecular structure and enzyme expression. Co-metabolism of chlorophenols has normally studied in the presence of other carbon and nitrogen source such

as glucose, sodium acetate, peptone, yeast extract [11]. The cometabolism of chlorophenols has also been studied in the presence of phenol, and other lower chlorinated phenolic compounds [14, 18]. It was observed that the degradation of higher chlorophenols increases in the presence of phenol and lower chlorophenols due to structural similarity, lower toxicity and enzyme expression [18, 19]. It has been reported that the presence of secondary carbon source and lower phenolic compounds contributes the biomass growth that leads to higher degradation rate [18, 19].

Another important parameter is the use of mixed microbial consortium for complete mineralization of chlorophenols. The single bacteria do not secrete all the enzymes requires for complete degradation of compounds. But microbial community contains a specific group of microbes that interact with each other and helps in complete mineralization of recalcitrant compounds. There are two different types of microbial consortium studied, one defined microbial consortium and second undefined microbial consortium. The activated sludge is called as the undefined mixed consortium that used for common wastewater treatment plants. However, sometimes the sludge microorganism doesn't contain all the needed for degradation of specific recalcitrant compounds. These microbial consortium present in the sludge and soil can be acclimated to specific compounds for improved biodegradation. Defined mixed consortium contains the specific microorganisms grew together for degradation of specific compounds. Known microorganisms, able to degrade specific compounds, are mixed to form defined consortium that degrade the mixture of specific compounds simultaneously. Kim et al., (2002) have reported the complete biodegradation of phenol, 4-chlorophenol, and 2,4,6-trichlorophenol mixture by a defined mixed culture of *P. testosteroni* CPW301 and *P. solanacearum*TCP114 [17]. Bae et al., (1997) has isolated two different pure cultures thatwere able to degrade the only selective substrate. *Pseudomonas* sp. TCP114 was able to degrade 2,4,6-TCP and phenol while *Arthrobacter* sp. CPR706 was only able to degrade 4-CP. When two bacteria were mixed, the resulting defined consortium was able to degrade all three chlorophenols simultaneously [20]. It has also been reported that undefined mixed consortia such as traditional activated sludge may frequently produce undesirable toxic metabolites that severely inhibit the growth of the chlorophenol-utilizing microorganisms in the mixture [20]. While defined mixed cultures are known to mineralize the compounds completelyalong with metabolites.

Biodegradation kinetic of chlorophenols is another important aspect to be studied for accurate prediction of effluent quality from treatment plants. Determination of biokinetic parameters is important for understanding the mechanism of bioreaction, for design and operation of the bioreactor and to define the optimal conditions for the removal of chlorophenol in biological treatment systems. Andrews's substrate inhibition model is most commonly used to describe the self-inhibitory effect of substrate on its transformation. Most of the biodegradation kinetics study has been performed for degradation of chlorophenols by pure culture. While for mixed culture, more information on biodegradation kinetics requires.

Removal of chlorophenols in bioreactors is another aspect that require detail evaluation. Attention has been given to study and modify the different bioreactors such as packed bed reactor, fluidized bed reactor, upflow anaerobic sludge blanket, combined

anaerobic and aerobic treatment and membrane reactor to enhance the performance of wastewater treatment containing lower to higher chlorophenols [21-23]. Attached biomass or supportive biofilm has an advantage of the high cell density and high specific biofilm surface area leading to high volumetric removal rate [24]. The optimization of different parameters is require for effective treatment of wastewater containing xenobiotic compounds in a bioreactor. These parameters include the hydraulic retention time, biogenic substrate concentration, substrate concentration, loading rate, airflow rate for the aerobic process, etc. Understanding of these different aspects has significant implications for effective treatment of chlorophenols present in the environment. A better understanding of the role that microorganisms play in the biodegradation and removal of chlorophenol compounds from the environment could facilitate further research for the effective implementation of bioremediation technologies for the contaminated sites. Specific treatment proficiency can enable removal of chlorophenols from the environment and subsequently their ecotoxicological effects.

1.2 Objectives

The proposed research work is based on the fact that the isolation source and acclimatization process have an impact on microorganism's potential for degradation of specific compounds. Also, the cometabolism of chlorophenols by pure and mixed microbial consortium is an important aspect that will help in *in-situ* bioremediation of the environment. The utilization of bioreactor for biodegradation of chlorophenols requires the understanding of the effect of different parameters such as hydraulic retention time, loading rate, and biogenic substrate concentration. The use of biofilm or attached microorganism for bioreactor would enhance the performance of the bioreactor. In this context, the specific objectives of the proposed research work can be listed as:

- Enrichment, isolation and selection of most potent microorganism capable of degrading chlorophenols from the contaminated site.
- The identification of the selected microorganisms – Physiological, Biochemical assays, 16S r-DNA analysis.
- To investigate the biodegradation, kinetic and cometabolism of chlorophenols by the pure single microorganism and by the mixed microbial consortium.
- To develop efficient batch process – optimization of process parameter such as temperature, pH, addition of carbon & nitrogen source, etc.
- To investigate the continuous removal of chlorophenols in the packed bed biofilm reactor (PBBR) and airlift inner loop reactor (ALR) by the pure and mixed microorganisms.
- To investigate the effect of different parameters such as hydraulic retention time, initial substrate concentration, loading rate and biogenic substrate concentration on removal efficiency of PBBR and ALR.

1.3 Layout of thesis

The whole thesis is organized into four main chapters as mentioned below followed by reference and conclusion section.

Chapter 1: Introduction

This chapter provides the introductory view and the central hypothesis of the research work carried out in this thesis. Also, it conveys the importance of the work, gaps and specific objectives of the work.

Chapter 2: Literature review

This chapter gives the literature available that supports the given present work. This chapter mainly focuses on the subjects of the research that have taken place previously, the research fallouts and possible ideas of research that can be incorporated in this study.

Chapter 3: Materials and methods

In this chapter, the different materials and methods employed in the present work have been mentioned. It gives the technical information about the detection and quantification of chlorophenols and its metabolites. The details methods of enrichment, isolation, and selection of the microbial strains used in the present work are explained. Also, the biodegradation experiments carried out using pure, and mixed consortium in the batch reactor are explained. The growth kinetic and biodegradation kinetic of chlorophenols are explained in detail. This chapter also gives the information on the three customized bioreactor including two packed bed biofilm reactor (PBBR) of 0.45 L and 0.75 L working volume and one airlift inner loop reactor (ALR) of 12 L working volume, build for the continuous biodegradation of chlorophenols in the lab.

Chapter 4: Results and discussion

This chapter is divided into four different sections according to the work done. The first section starts with the isolation and selection of potent bacterial strains. It also contains the characterization of selected microbial strains such as morphological, biochemical and 16S rDNA analysis.

The next sections emphasize on the biodegradation of the six different chlorophenols including 2-CP, 3-CP, 4-CP, 2,4-DCP, 2,4,6-TCP and PCP by three pure isolated bacterial cultures. The growth kinetic and biodegradation kinetic study of all six chlorophenols by three individual strains are explained. It also emphasizes on the optimization of four different experimental parameters such as pH, temperature, inoculum size, and ammonium sulphate as a nitrogen source, using the response surface methodology to achieve the maximum biodegradation of chlorophenols. Also, the cometabolism of various chlorophenols by single bacterial strains is interpreted in detail.

The third section details about the biodegradation of chlorophenols and its co-metabolism by the two different mixed microbial consortia, one defined consortium and the

other one undefined consortium. The biodegradation kinetics is also studied using Andrews's substrate inhibition model for the biodegradation of chlorophenols by the mixed consortium.

The final part of the chapter focused on the continuous degradation of chlorophenols in the bioreactor. In the first packed bed biofilm reactor, the biodegradation of 2,4-dichlorpohenol by two different isolated strains was studied. In the second packed bed biofilm reactor, the biodegradation of 3-chlorophenol and 4-chlorophenol by the defined mixed microbial consortium was studied individually. In the third reactor i.e., airlift inner loop reactor, two different studies have been conducted. First, the biodegradation of 4-chlorophenol by the mixed microbial consortium (undefined) was explained with themetabolic pathway. Second, the biodegradation of mixture of different chlorophenols including 2-chlorophenol, 3-chlorpohenol, 4-chlorophenol, 2,4-dichlorophenol, and 2,4,6-trichlorophenol was conducted. In all the three bioreactor studies, the effect of hydraulic retention time, initial substrate concentration or loading rate and effect of biogenic substrate concentration on removal efficiency of chlorophenols in bioreactors during continuous mode was evaluated extensively.

The last part of the thesis focused on the possible future aspect of the present research work that may be further extended

CHAPTER – II

LITERATURE REVIEW

LITERATURE REVIEW

2.1. Properties of Chlorophenols

Chlorophenol is an aromatic ring structure comprising of benzene ring, a hydroxyl group (-OH) and chlorine ion (Cl^-). Chlorocresols are also considered as chlorophenols [25]. All chlorophenols are solid at room temperature except 2-CP, which is liquid. There are 19 different congeners of chlorophenols depending on number and position of chlorine ion. The common structure of the chlorophenols has been presented in figure 2.1.

Where, x = 1 to 5

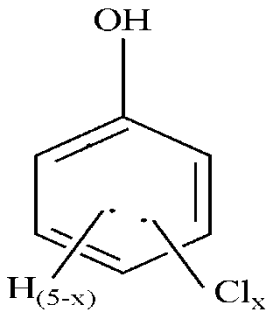


Figure 2.1: The common structure of the chlorophenol compounds.

Physico-chemical properties of some selected chlorophenols havebeen presented in

Table 2.1. Some of the points can be summarized as below:

- With the increase in the degree of chlorination, their solubility in water decreases and their N-octanol/water partition coefficient (Ko/w) increases indicating a propensity for higher chlorophenol to bioaccumulate.
- They are weak acid and at pH>pKa, a phenoxide salt forms, which is highly soluble in water [10].
- Increasing chlorination increases the tendency of chlorophenols to partition into sediments and lipids and leads to bioaccumulation.

2.2. Source and Toxicity of Chlorophenols

Chlorophenols and its derivatives are persistent, mobile and present in all environmental compartments. Some common source of chlorophenols contamination can be summarized as –

- The food chain is responsible for 99.8% of human exposure to people not occupationally or intentionally exposed [26]
- Accidental spills and leaching from Lumber

- Chlorine bleaching process in paper & pulp mills
- Hazardous waste disposal sites
- During chlorination of wastewater and drinking water for disinfection purpose
- Storage tanks
- Municipal landfills.
- Biodegradation/breakdown of pesticides and herbicides
- During the combustion of organic matters

Chlorophenol compounds may cause some histopathological alteration, mutagenicity genotoxicity, carcinogenicity and immunogenicity in humans and other organisms [4, 5]. The International Agency for Research on Cancers (IARC) have classified 2,4,6-TCP, 2,4,5-TCP and Pentachlorophenol as group 2B possible carcinogen to human [6, 7, 27, 28]. It has also been found that dioxin, furan and PCB levels were higher than background serum among former TCP and PCP workers and also in individuals living in log homes [29]. Chlorophenols interfere with the metabolizing enzymes which lead to increased respiration rate [8]. Daniel et al. (2001) manifested the possible relationship between blood levels of PCP and immune function. It's been concluded that increased blood levels of PCP were associated significantly with cellular and humoral immune-deficiencies. Also, the patients suffered from frequent respiratory infections and general fatigue [9]. Toxicity of chlorophenols in humans has been obtained from animal studies as toxicity data of chlorophenols on humans are yet to be probed [3]. Table 2.2shows the distribution and exposure level of various chlorophenols found in the environment.

Acute poisoning by chlorophenols may result in hyperthermia, profuse sweating, convulsions, elevated heart rate, breathing difficulty, restlessness, agitation, muscle twitching, tremors, epigastric tenderness, and leg pain. Chronic poisoning is vague and varies considerably in different reports but can include chloracne, anorexia, weight loss, obstinate headache, anxiety, indications of liver damage and neurological effects. [6] Sudden death may occur as a result of cardiac arrest. [8]

The toxicity of the chlorophenol varies with the degree of chlorination and lipophilicity. Environmental Protection Agency (EPA) has set an enforceable regulation for pentachlorophenol, called a maximum contaminant level (MCL) at 1 ppb [30]. The recommended maximum acceptable concentration in drinking water quality and surface water for 2,4,6-TCP and PCP is 0.2 ppm and 9 ppb[31]. The EPA recommends that drinking water concentrations of 2-CP, 2,4-DCP, 2,4,5-TCP and 2,3,4,6-TeCP should not be more than 0.1, 0.3, 1 and 1 ppb, respectively and these are the levels EPA recommends in order for chlorophenols to be lower than levels that can be tasted [32].The taste thresholds in water for 2-CP, 2,4-DCP, and 2,4,6-TCP are 0.1, 0.3, and 2 ppb and the odor thresholds are 10, 40, and 300 ppb, respectively [25, 31].

Table 2.1: Physico-chemical properties of chlorophenol compounds

Compound	MP (^{0}C)	BP (^{0}C)	Density (g/mL)	Log K_{OW}	Log K_{OC}	Solubility in water (g/L)	Vapour Pressure (mm Hg)	pKa	Henry's Low Constant (atm-m^3/mol)
2-CP	9.3	174.9	1.2634	2.17	1.25-3.7	28.5	0.99	8.49	6.8×10^{-6}
4-CP	43.2-43.7	220	1.2238	2.4	1.2-2.7	27.1	0.23	8.85	9.2×10^{-7}
2,4-DCP	45	210	1.383	3.2	2.42-3.98	4.5	0.14	7.68	4.3×10^{-6}
2,4,5-TCP	67	235	1.678	3.72	2.55-3.98	0.948	0.05	7.43	5.1×10^{-6}
2,4,6-TCP	69	246	1.49	3.69	1.94-3.34	0.8	0.03	7.42	5.7×10^{-6}
2,3,4,5-TeCP	116-117	Sublime	1.67	4.8	2.9-4.14	0.166	0.0059	6.96	1.3×10^{-6}
2,3,4,6-TeCP	70	64	1.83	4.45	3.2-4.21	0.183	0.0059	5.38	3.6×10^{-6}
2,3,5,6-TeCP	115	288	1.84	4.9	3.88-4.9	0.1	0.0059	5.48	2.2×10^{-6}
PCP	190	310	1.987	5.01	1.2	0.014	0.0002	4.74	na

Source: [32]; (**K_{OW} -** N-octanol/water partition coefficient**; K_{OC} –** Organic carbon partition coefficient**; na-** not available)

Table 2.2: Presence and exposure level of chlorophenols into the environment

Compound	Source	Concentration level	Reference
4-CP	Urine	2.1 – 265 µg/L	[33]
24-DCP	Urine	2.2 – 450 µg/L	[34]
25-DCP	Urine	0.4-1550 µg/L	[33]
	Urine	2.2 – 8700 µg/L	[34]
2,4,5-TCP	Drinking water	0.330 – 0.350 µg/L	[35]
	Tape water	0.486 µg/L	[35]
2,4,6-TCP	Urine	1975 – 2331 µg/L	[36]
2,3,5,6-TeCP	Tape water	0.221 µg/L	[35]
2,3,4,6-TeCP	Urine	788.8 – 4013.6 µg/L	[36]
	Adipose tissue	2 – 31 µg/kg	[37]
	Urine	0.3 – 5.8 µg/L	[33]
PCP	Urine	53.2 – 239.4 µg/L	[36]
	Urine	0.6 – 19.1 µg/L	[33]
	Urine	1.5 – 55 µg/L	[34]
	Adipose tissue	2 – 31 µg/kg	[37]
	Liver	4 µg/kg	[37]
	Blood	>20 µg/L	[9]
	Blood	>20 µg/L	[9]
	Blood Serum	84.9 µg/L	[38]
	Urine	13.8 µg/L	[38]

2.3. Microbial Degradation of Chlorophenols

Most of the synthetic organic compounds (SOC) such as chlorophenols are alien to nature and therefore recalcitrant. The use of microorganism for degradation of such SOCs have some limitation, but it gives the complete mineralization if provided with the suitable physiological environment. The biological process indeed adds up following advantages over the physicochemical process.

- Chemicals' requirement for the whole treatment process is reduced.
- Low capital and operating costs compared to alternatives.
- An eco-friendly and cost-effective alternative to conventional techniques.
- Reduction of aquatic toxicity.
- Efficient at lower levels of contamination.

There are various factors that affects the biodegradation of SOCs such as: pH, temperature, and the presence of nutrient and electron acceptors. The molecular structure of the compounds has also affect the biodegradation of SOC. Substitution of the SOC makes them more resistant to biodegradation than the unsubstituted parent compound. Chlorophenols are more recalcitrant than phenol. Also, the number and position of the chloride ions or halogen on benzene ring have a greater effect on the biodegradability of the compounds. Substitution at one position may have a lower effect on degradation than at other position. In case of monochlorophenol, 2-CP (-*ortho* substitution) can easily degraded than other 3-CP (-*meta*) and 4-CP (-*para* substitution). The study of this structure-biodegradability relationship is significant in a deduction of biodegradability of the compounds from molecular structure. The solubility of the compounds is another important factor that affects the biodegradation. With increasing chlorination, the solubility of the chlorophenols decreases in the medium resulting in lower bioavailability to the microorganism and ultimately decreased the rate of biodegradation.

The presence or absence of oxygen is also an important factor that has a large impact on the biodegradability efficiency. Some enzymatic steps such as dioxygenase require the presence of oxygen for its action whereas reductive dechlorination (anaerobic biodegradation) requires the absence of oxygen. Understanding of the metabolic pathway for SOC degradation is significant for designing wastewater treatment process for the complete mineralization of the compounds. Both aerobic and anaerobic process are important for complete degradation of the chlorophenols which has been discussed in the next section.

2.3.1. Aerobic biotransformation of chlorophenols

Many aerobic microorganism including bacteria, fungi and algae present in the natural habitats utilize chlorophenols as a sole carbon and energy source. Aerobic biodegradation is usually preferred for lower chlorophenols such as mono and di-chlorophenol and sometimes tri-chlorophenols also. Aerobic biodegradation or dechlorination become less effective for higher chlorophenols as compared to anaerobic biodegradation.While, for the treatment of

higher chlorophenols such as tri, tetra, and pentachlorophenol, an anaerobic biodegradation is more effective.

Complete mineralization of chlorophenols requires two criteria. First, the removal of chloride ion from the aromatic ring followed by the breakdown of aromatic ring or benzene ring. Aerobic biodegradation of chlorophenols takes place in two steps. The first step is an initial attack by monooxygenase or dioxygenase enzyme on chlorophenols followed by dehalogenation of resulting compound by oxygenolytic, hydrolytic and reductive mechanisms. Dioxygenase attack initiates the degradation process of lower chlorophenols (CP and DCP) without dehalogenation to yield the corresponding chlorocatechol. The dehalogenation proceeds after oxidative ring cleavage [39, 40]. While in case of higher chlorophenols (TCP, TeCP, and PCP) aromatic ring is cleaved only after dehalogenation of all or most chlorine substituent, since the halogen atoms deactivate the aromatic nucleus to electrophilic attack by dioxygenases [39].

The aerobic biodegradation of monochlorophenols follows the two main pathways, *ortho* and *meta* fission. The first step in the degradation of MCPs involves their transformation into chlorocatechol (CC). 2-chlorophenol (2-CP) and 3-chlorophenol (3-CP) are being converted to 3-chlorocatechol (3-CC) while 4-chlorophenol (4-CP) is being converted to 4-chlorocatechol (4-CC) [41, 42]. The second step is the ring cleavage of chlorocatechol by dioxygenases. The aromatic ring cleavage of CC occurs via either *ortho* or *meta* pathway [43]. The enzyme catechol 1,2-dioxygenase are responsible for the degradation of chloroaromatic compounds via *ortho*- cleavage pathway [40]. The *meta*- cleavage of 3-CC by catechol 2,3-dioxygenase is results in dead-end pathway due to the generation of suicide or dead-end metabolites [44-46]. The *meta*-cleavage of 4-CC results in the production of 5-chloro-2-hydroxymuconic semialdehyde (5-CHMS) which has been reported to be a dead-end metabolite [42, 47]. But recent studies have shown the further degradation of 5-CHMS, indicating complete degradation of 4-CP via *meta*- fission pathway [40, 48, 49]. After ring cleavage, chlorine substituents are removed by dehalogenation process and products further goes into the TCA cycle and are finally converted to H_2O and CO_2.

There are some aerobic microorganisms which degrade 4-CP via the novel route, where 4-CP is converted into hydroquinone. A newly isolated *Arthrohacter ureqfaciens*CPR706, degrade 4-CP via a new pathway, in which the chloro-substituent was eliminated in the first step, and hydroquinone was produced as a transient intermediate [50]. A similar result was observed by *Arthrobacter chlorophenolicus* A6 strain which degrade 4-chlorophenol via hydroxyquinol intermediate [51].

The degradation of polychlorinated phenols (TCP, TeCP, and PCP) by aerobic microorganism takes place by initial oxygenase attack to yield chlorohydroquinones. The aromatic ring is cleaved only after dehalogenation of all or most chlorine substituent [39, 52, 53]. The biodegradation pathway of polychlorinated phenols have been studied in many bacteria including *Sphingomonas sp.,Rhodococcus sp., Flavobacterium sp., Arthrobacter sp., Mycobacterium sp., Ralstonia sp*.,[52-56] and fungi such as *Mucor plumbeus, Phanerochaete chrysosporium, Bjerkandera adusta and Anthracophyllum discolor* [57-59].

*Sphingobium chlorophenolicum (*formerly known as *Sphingomonas chlorophenolica)*is the best-studied soil microorganism which can grow on PCP as a sole carbon source. Studies on *S. chlorophenolicum* have revealed PCP degradation pathway and intermediate products [53, 56, 60]. In the degradation of PCP using this microorganism, the pathway begins with the conversion of PCP to tetrachlorohydroquinone (TeCHQ). The next two steps are reductive dehalogenation in which chlorine atoms are replaced by hydrogen to form first trichlorohydroquinone (TCHQ), and then 2,6-dichlorohydroquinone (DCHQ) [53]. 2,6-dichlorohydroquinone (DCHQ) is subjected to ring cleavage by dioxygenase, producing 2-chloromaleylacetate (2-CMA) which is subsequently converted to maleylacetate by reductive dehalogenation and then a subsequent reduction of malyelacetate to 3-oxoadipate [61, 62]. Dai et al. (2003) proposed a modification of PCP degradation pathway in *S. chlorophenolicum.* They proposed the first step in PCP degradation is the conversion of PCP to tetrachlorobenzoquinonewhichis subsequently reduced by NADPH-dependent reduction to tetrachlorohydroquinone[56]. Similar PCP degradation pathway were also proposed with other bacterial species such as *Arthrobacter sp.* ATCC 33790 [54] and *Rhodococcus sp.* CP-2 [63].

Padilla et al. (2000) studied the catabolic pathway of 2,4,6-TCP in *Ralstonia eutropha* JMP134, *Ralstonia eutropha*JMP222 and *Ralstonia* sp. PZK. The pathway is initiated by conversion of 2,4,6-TCP to 2,6-dichlorohydroquinone (2,6-DCHQ). The next three steps involve reductive dehalogenation of 2,6-DCHQ to form 6-chlorohydroxyquinol, 2-chloromaleylacetate, and maleylacetate [52].

The catabolic pathway of PCP and 2,3,5,6-tetrachlorophenol degradation in *Rhodococcus sp.* CP-2 and *Rhodococcus chlorophenolicus* PCP-I involves para-hydroxylation into TeCHQ [63, 64], followed by a second hydroxylation and three reductive dechlorination reaction to form non-chlorinated product 1,2,4-trihydroxybenzene (1,2,4-THB). All the chlorine substituents are removed before ring cleavage. 1,2,4-THB is ring-cleaved with subsequent degradation to CO_2 showing complete mineralization of PCP and TeCP.

Reddy and Gold (2000), proposed a catabolic pathway for PCP in *P. chrysosporium*under nitrogen-limiting, secondary metabolic condition. The pathway is initiated with the oxidative dechlorination of PCP to tetrachloro-1,4- benzoquinone (TCBQ) by either lignin peroxidase (LiP) or manganese peroxidase (MnP). The second step is the reduction of the TCBQ to tetrachlorodihydroxybenzene, which can undergo four successive reductive dechlorination to produce 1,4-hydroquinone, which is *o*-hydroxylated to form the final aromatic metabolite, 1,2,4-THB. The alternative step in the pathway is the conversion of the TCBQ, either enzymatically or non-enzymatically, to 2,3,5-trichlorotrihydroxybenzene, which undergoes successive reductive dechlorinations to produce 1,2,4-THB. Apparently, all five chlorine atoms are removed from the substrate prior to ring cleavage. The 1,2,4-THB produced in each pathway is ring-cleaved with subsequent degradation to CO_2[57]. The similar pathway is also reported for 2,4,6-TCP in *P. chrysosporium*under nitrogen-limiting, secondary metabolic condition [65].

Some researcher reported that PCP and TeCP degradation pathway first start with the o-methylation of PCP and TeCP to form corresponding chlorinated anisole. Rubilar et al. (2007) reported that the biodegradation of PCP by *Anthracophyllum discolor* is initiated by the production of pentachloroanisole (PCA) which is completely degraded by subsequent serial reactions of demethoxylation, carboxylation, reduction, and methylation [58]. Various bacteria such as *Rhodococcus* sp. and *Mycobacterium* sp. has also been reported to show o-methylation of PCP [66, 67].

2.3.2. Anaerobic biotransformation of chlorophenols (Halo respiration)

Certain anaerobic microorganisms have the potential to utilize chlorophenols as carbon and energy source. Reductive dechlorination is the prominent mechanism for degradation of chlorophenols under anaerobic condition. Sometimes, chlorophenol compounds serve as a terminal electron acceptor in addition to being carbon source, known as halorespiration or dehalorespiration, the process coupled to growth and energy yield [68, 69]. It has been reported that anaerobic biotransformation or reductive dechlorination is more efficient for higher chlorinated compounds and become less efficient with dechlorination of the compounds.

Biotransformation of PCP by sequential *ortho*-dechlorination to produce 2,3,4,5-TeCP and 3,4,5-TCP in the presence of acetate by anaerobic digester sludge has been reported[70]. However upon acclimation to PCP over a period of 6 monthsthe methanogenic consortium removed chlorines from the *ortho*, *meta*, and *para* positions of PCP and its reductive dechlorination products. A similar result was also reported in which PCP was reductively dechlorinated at *para*, *ortho* and finally *meta* position by an enriched anaerobic consortium in the presence of secondary carbon sources such as glucose and sodium formate [71]. Bouchard et al. (1996) isolated the first anaerobic bacteria strain *Desulfitobacterium frappieri*PCP-l which can dechlorinate PCP to 3-CP [72]. PCP is completely degraded by a methanogenic consortium in medium containing glucose and formate as complementary carbon sources. These PCP and TeCP are sequentially dechlorinated first at *ortho*, *para* and then finally at the *meta* positions. The catabolic pathway of 2,4,6-TCP mineralization by reductive dechlorination has also been reported, where TCP was reductively dechlorinated at *ortho*-positionto successively generate 2,4-DCP, 4-CP or 2-CP [29, 73].

Reductive dehalogenation of chlorophenols coupled to the reduction of nitrate, ferric ion, sulfate and other alternate electron acceptor have been reported in many studies. The biodegradation of 2-CP, 3-CP, 4-CP and 2,4-DCP have also been examined under a sulfate-reducing condition with an estuarine sediment inoculum. [74] Sulfidogenesis was confirmed using $^{35}SO_4^{2-}$ radiotracer technique by the recovery of radiolabeled sulfide in the Zn acetate traps. Phenol and 2-, 3-, and 4-CP were degraded in Hudson River sediments, with the concomitant reduction of Fe^{3+} to Fe^{2+}. The resultant production of Fe^{2+} with the degradation of these compounds indicates the complete mineralization [75]. The evidence of chlorophenols degradation under nitrate reduction condition has also been reported. The dechlorination of 2-CP under denitrifying condition was reported with a specific growth rate of 0.0139 d^{-1}. The concomitant and stoichiometric consumption of nitrate during 2-CP

degradation with no detection of dechlorination intermediate, suggesting that 2-CP degradation was coupled to respiratory denitrification [76]. Similarly, Jianlong et al. (2001) demonstrated the anaerobic degradation of 4-CP with the stoichiometric release of chloride under denitrifying conditions [77]. The degradation of 3-CP [78] and 3-chlorobenzoate [79] by an enriched microbial consortium in an anaerobic continuous-flow fixed-bed column reactor under circum-denitrifying conditions have been evidenced.

Halorespiration or dehalorespiration is a process in which the dechlorination of the chloro-aromatic compound is coupled to growth and energy yield in several anaerobic bacteria. In halorespiration chlorinated organic compound is used as a terminal electron accepter during dechlorination process by anaerobic bacteria [69]. Reductive dechlorination process is exergonic and microorganisms can potentially conserve energy for growth by routing electrons in the form of H_2 from anaerobic environments to halogenated organic compounds as electron acceptors [68]. Dolfing, (1990) confirmed the hypothesis that under anaerobic condition reductive dechlorination of 3-chlorobenzoate is coupled to energy yield. He evidenced this hypothesis by observing the increase in cell growth and ATP level in the cell [80]. Several bacterial strains such as *Desulfomonile tiedjei* DCB-1 [81], *Desulfitobacterium dehalogenans* JW/IU-DC1 [82], *Desulfitobacterium hafniense* DCB-2 [83], *Desulfitobacterium chlororespirans* Co23 [84],*Desulfovibrio dechloracetivorans* SF3 [85], *Anaeromyxobacter dehalogenans* 2CP-C [86],have been isolated and characterized which are capable of halorespiration. The halorespiration in all of these bacteria was shown by measuring the increase in protein yield and ATP formation. Common electron donors used in most halorespiration processes are pyruvate, formate, lactate, H_2, acetate and ethanol. Most bacteria have been shown to dechlorinate chlorophenols via *ortho*-cleavage pathway. However, Bouchard et al. (1996) isolated the *Desulfitobacterium frappieri* PCP-l from a methanogenic consortium with the ability to reductively dechlorinate chlorophenols at *ortho, meta* and *para*-position[72]. The strain *Desulfomonile tiedjei*DCB-1 has been shown to dehalogenated the *meta*-chlorine substituent from chlorophenols in the presence of 3-chlorobenzoate which act as an inducer[81]. Van de Pas et al. (1999) purified and characterized the catalytic subunit of the *o*-CP dehalogenase from *Desulfitobacterium dehalogenans*. The purified dehalogenase catalyzed the reductive dechlorination at the *ortho* position of 3-chloro-4-hydroxyphenylacetate, 2-CP, 2,3-DCP, 2,4-DCP, 2,6-DCP, PCP, and 2-bromo-4-CP with reduced methyl viologen as an electron donor [87].

2.4. Cometabolism

Chlorophenols are toxic in nature and have an inhibitory effect on the microorganism. There is some microorganism that utilize the chlorophenols (SOC) as sole carbon and energy source. However, this is not always the case for other microorganism. Some microorganism utilizes the SOC without using the compounds as a source of energy or growth. Even the metabolism of the substrate that support the growth occurs by some bacteria without incorporating the carbon into their cells [88, 89]. This is referred as cometabolism. Basically, the microorganism utilizes one substrate for growth and in the process simultaneously transform non-growth substrate using enzymes induced for the utilization of the growth substrate [90]. Co-metabolism of SOC such as chlorophenols are important in understanding

the *in-situ* bioremediation, synergetic of the biodegradation and multi-substrate degradation process that has encountered in the *in-situ* environment. There are more than one chlorophenol congeners are present together along with other aromatic compounds and nutrient in the environment and industrial effluent. The presence of other toxic compounds and nutrient has a great influence on the biodegradation of chlorophenols. Biodegradation of some toxic compounds is observed to increase or decrease in the presence of other less toxic compounds [18, 19].

Co-metabolism of chlorophenols was normally studied in the presence of other carbon and nitrogen sources such as glucose, sodium acetate, sodium glutamate, peptone, yeast extract [16, 91-93]. The cometabolism of chlorophenols has also been studied by some researchers in the presence of phenol, and other lower chlorinated phenolic compounds [14, 18, 90, 94, 95]. The presence of nutrient and lower phenolic compounds has positive as well as negative effects on the degradation of higher chlorophenols depending on the molecular structure, enzyme expression, interaction between growth and non-growth substrate and presence of metabolites [18, 19].The degradation of higher chlorophenols has been found to increase in the presence of phenol and other lower chlorophenols due to their structural similarity and lower toxicity of the compounds. Also, their presence induces the biomass growth that leads to higher degradation rate [18, 19]. However, converse effects are also reported in the literature.

Wang et al., (2014) reported that phenol could induce the enzyme required for 4-CP biodegradation in *P. putida* LY1. The result showed that the strain *P. putida* LY1 could not grow on 4-CP as sole carbon source. Cometabolic study showed that at high phenol to 4-CP ratio, phenol was first transferred to metabolites that could be utilized by the bacteria for as growth substrate [90]. Hao et al., (2002) reported **s**ynchronous 4-CP and phenol degradation by *Acinetobacter* sp. at sufficiently high initial phenol:4-CP concentration ratio (200:50 mg/L) [96]. Kim and Hao (1999) reported that critical ratio between phenol and chlorophenol was necessary for complete biodegradation of 3-CP and 4-CP and below this ratio, both 3-CP and 4-CP were shown partial degradation. Here the strains utilize the phenol as growth substrate (primary substrate) and the enzymes induced by growth substrate acts on nongrowth substrate (secondary substrate) and add alteration to it or partially degrade the compound of interest. These induced enzymes does not guarantee the complete degradation of secondary substrate [97]. Similarly, wang and Loh (2000, 2001) reported that the transformation of 4-CP by *P. putida* was enhanced by phenol in addition to sodium glutamate (readily degradable substrate). They reported that both phenol and sodium glutamate as growth substrate playeddifferent role in the complete degradation of 4-CP. Utilization of phenol as a specific growth substrate induces the enzymes that responsible for phenol and 4-CP transformation. While the presence of sodium glutamate increases the specific growth rate and generation of more active microbial cells [98, 99].

Quan et al., (2003, 2004) studied the biodegradation of phenol and 2,4-DCP by *Achromobacter* sp., immobilized on honeycomb like ceramic as a carrier in an airlift inner-loop bioreactor. The results showed that presence of phenol inhibited the biodegradation of

2,4-DCP and caused the major carbon shift from 2,4-DCP (higher toxic) to phenol (lower toxic) [21, 100].

Wang et al., (2000) isolated *Pseudomonas spp*. strain 01, *Pseudomonas spp*. strain 02 and *Agrobacterium spp.* From the mixed culture to study the biodegradation of 2,4,6-TCP in the presence of primary substrate. Whether in suspended or immobilized forms, all strains have poor removal efficiencies of 2,4,6-TCP. The presence of primary substrates such as 2-CP, 3-CP, 4-CP, and 2,4-DCP could not assist in the removal of 2,4,6-TCP. However, the addition of 200 mg/L of phenol enabled the immobilized *Pseudomonas spp.* strain 01, and *Pseudomonas spp*. strain 02 to achieve 65% and 48% removal of 2,4,6-trichlorophenol, respectively [101].

Durruty et al., (2011) studied the multisubstrate degradation of chlorophenols by a mixture of *Pseudomonas aeruginosa* and a novel *Acromobactor* sp. capable of using PCP, 2,4,6-TCP and 2,3,5,6-TeCP as sole carbon and energy sources. Batch experiments were conducted with each chlorophenols separately and in mixtures of PCP + 2,4,6 TCP, PCP + 2,3,5,6 TeCP, and PCP + 2,4,6 TCP + 2,3,5,6 TeCP. The results indicated the simultaneous degradation of CPs is a key factor contributing to the improvement of PCP degradation. Also, the addition of less recalcitrant chlorophenols such as 2,4,6-TCP improves total chlorophenols degradation in comparison to PCP as the only source of carbon and energy [18].

Liu et al., (2006) explained the difference between the degradation of single chlorophenols and their mixture using the cometabolism principle. Three stable chlorophenols, 3,5-DCP, 3,4,5-TCP, and 2,3,5,6-TeCP, which showed a total resistance to the biodegradation potential of a PCP degrading bacterial culture, were found to be biodegradable using the cometabolic technique. The result showed that when a low chlorinated 3-MCP used as cometabolite, only the medium low chlorinated 3,5-DCP was biodegraded while the culture did not degrade 3,4,5-TCP and 2,3,5,6-TeCP. However, all three stable chlorophenols were rapidly biodegraded when a highly chlorinated PCP used as cometabolite [102].

2.5. Mixed consortium

Single bacterial strain can degrade the toxic compounds completely if provided the feasible environment and presence of primary growth substrate. However, sometimes pure strain doesn't possess or express all the enzymes requires for complete mineralization of toxic compounds. They only express the enzymes that acts on parent compounds and produce the intermediate metabolites. These intermediate metabolites remains in the medium unutilized because of the lack of enzymes requires in the metabolic pathway. Also, use of pure strains in the *in-situ* environment is impractical as the dominance of other strains over the special strains that are better fitted for the degradation of target compound.

In nature or engineered system, microbial communities are responsible for the complete mineralization of the toxic compounds. The several species present act together in a coordinated way within the microbial community that lead to complete degradation of SOC.

Also, mixed microbial consortium does not require the presence of other primary growth substrate such as phenol or carbon source for complete degradation or induction of enzymes for the transformation of target toxic compound. Although sometimes mixed consortium such as activated sludge lacks the specific bacterial strains, that act on target compounds. But these problem can be eliminated by acclimation of the consortia.

Bae et al., (1997) has isolated two different pure cultures whichwere able to degrade only selective substrate. *Pseudomonas* sp. TCP114 was able to degrade 2,4,6-TCP and phenol while *Arthrobacter* sp. CPR706 was only able to degrade 4-CP. When two bacteria were mixed, the resulting defined consortium was able to degrade all three chlorophenols simultaneously [20]. Kim et al., 2002 has reported the complete biodegradation of phenol, 4-chlorophenol, and 2,4,6-trichlorophenol mixture by a defined mixed culture. The presence of 2,4,6-TCP inhibited the degradation of phenol and 4-CP by a single pure strain, but when the strain that has ability to degrade 2,4,6-TCP was added to the mixture, the resulting mixture was able to degrade all three compounds [17].

Farrell and Quilty (1999) examined a mixed microbial community, specially designed to degrade a wide range of substituted aromatic compounds, for its ability to degrade mono-chlorophenols as sole carbon source in aerobic batch cultures. The mixed culture degraded 2-CP, 3-CP, and 4-CP (1.56 mM) via a *meta*-cleavage pathway. 2-CP and 3-CP were degraded via 3-chlorocatechol by the mixed culture. Further metabolism was toxic to cells as it led to inactivation of the catechol 2,3-dioxygenase enzyme upon *meta*-cleavage of 3-chlorocatechol resulting in incomplete degradation and an accumulation of brown colored polymers. While, degradation of 4-CP by the mixed culture led to an accumulation of 5-CHMS, the *meta* cleavage product of 4-chlorocatechol. The mixed culture further metabolized 5-CHMS with a stoichiometric release of chloride, indicating complete degradation of 4-CP by the mixed culture via a *meta*- cleavage pathway [40].

Herrera et al., (2008) assessed the biodegradation of 2-CP, 3-CP, and 2,4-DCP by *Bacillus* consortium isolated from the polluted soil. The author reported that degradation of 2,4-DCP by pure cultures of *Bacillus* strains was very low compared to when used as a consortium. Also, in the presence of NH_4Cl and KNO_2 nitrogen source, 2,4-DCP was variously degraded [11].

Acclimation is an important factor in biodegradation of recalcitrant compounds in wastewater. Acclimation in common term can be explained as prolonged contact of amicroorganism or the community to toxic compounds under favorable conditions. During the acclimation process, the microorganisms within the microbial community develop the ability to degrade recalcitrant or non-biodegradable compounds called adaptation. The time required for acclimation can vary from several hours to days depending the compound and its toxicity, molecular structure, surrounding environment, the presence of secondary substrates and microbial community. Adaptation is of two types either phenotypic or genotypic. In the phenotypic adaptation, the genetic information content remains unchanged but the degree of expression of certain genes is altered. Phenotypic adaptation may occur during short-term fluctuations in the environment. Genetic adaptation involves changes in the genetic

information of the cell via two different basic methods: mutation and recombination. When acclimated organisms are subjected to a different environment, they tend to adapt to the new situation and may lose the ability to grow at the expense of the original substrate. Phenotypic adaptations are temporary and, therefore, more rapidly lost than genotypic adaptations [103]. This process of acclimation and deacclimation of the microorganism has a great influence on the biodegradation of recalcitrant compounds whichare discharged intermittently.

Sahinkaya and Dilek (2005) studied the effect of acclimation on activated sludge for removal of 4-CP. The result showed that the toxicity of 4-CP on the culture decreased remarkably after acclimation. Also, the unacclimated culture was not able to remove 4-CP, but complete removal of 4-CP was observed with acclimated culture up to 300 mg/L [104]. Ye and Shen (2004) studied the effect of acclimation of sludge collected from Hangzhou citrate factory and Hangzhou municipal wastewater treatment plant for biodegradation of chlorophenols with or without the addition of sucrose. The results showed that acclimation with chlorophenols (2-CP, 3-CP, 4-CP, and PCP) can increase the degradation activity of anaerobic sludge and degradation rate of chlorophenolic compounds, and reduce the lag time. Also, the dechlorination activity of the acclimated sludge strongly depended on sludge source, microorganism population, and chlorophenol congener. 2-CP was more easily acclimated than 3-CP and 4-CP. Among the four tested compounds, 4-CP was the most difficult to be acclimated. The result showed that in the presence of sucrose, the degradation rate was higher than that with absence of sucrose which suggest that the addition of the external carbon source can stimulate the formation of acclimated sludge which could effectively degrade chlorophenols [105].

Microbial community acclimated to one recalcitrant compound may or may not able to utilize another recalcitrant compounds. Wiggings et al., (1987) suggested that there are a selection and a multiplication of specialized microorganisms during acclimation occurs [106]. Boyd and Shelton (1984) investigated the anaerobic biodegradation of mono and di-chlorophenols by fresh (unacclimated) sludge and by sludge acclimated to either 2-CP, 3-CP or 4-CP. The acclimated sludge, in general, gave patterns of degradation distinctly different from those of fresh sludge. The authors also evaluated the effect of cross-acclimation to monochlorophenols on biodegradation pattern. The result showed that sludge acclimated to 2-CP cross-acclimated to 4-CP but did not utilize 3-CP. This sludge also degraded 2,4-DCP. Sludge acclimated to 3-CP cross-acclimated to 4-CP but not to 2-CP. This sludge degraded 3,4-DCP and 3,5-DCP but not 2,3-DCP or 2,5-DCP. The specific cross-acclimation patterns observed for monochlorophenol degradation demonstrated the existence of two unique microbial activities that were in turn different from fresh sludge. The sludge acclimated to 4-CP could degraded all three MCP isomers and 2,4-DCP and 3,4-DCP. The active microbial population in this sludge appeared to be a mixture of populations present in the 2-CP and 3-CP acclimated sludge, both of which could utilize 4-CP [107].

2.6. Biotransformation of chlorophenols in the natural environment

The fate and transport of chlorophenols when enter into the natural environment, is governed by many physicochemical processes such as photo-degradation, biodegradation,

sorption, hydrolysis, oxidation. Various factors such as pH, temperature, humidity, chemical structure, number and position of chlorine atoms reflects the behavior, bioavailability and consequent degradation of chlorophenols [10]. Several studies have shown that microorganisms from soil, sediments, groundwater and sludge that have not been exposed to this type of pollutant can degrade chlorophenols[12, 108, 109]. **Table 2.3** represents the list of microbes present in the various parts of the environment having ability to tolerate and/or utilize the chlorophenols.

2.6.1. Biodegradation of chlorophenols in the soil

Indigenous microbial communities of unexposed soil have good potential for the bioremediation of chlorophenol contaminated soil. Influence of 2-CP, 2,4,6-TCP, and PCP on the indigenous microbial community of a previously unpolluted Mediterranean soil was reported. In the report,significant change in soil respirometric values and the bacterial community composition were observed at concentrations above 1000 mg/kg of2-CP and TCP, and above 100 mg/kg of PCP. There are 23 types of different bacterial strain isolated which has shown high resistance to chlorophenols [110]. Sanchez et al. (2004) reported that the endogenous microbial community of previously unexposed forest soil effectively degraded 2,4,6-TCP up to 5000 mg/L, showing significant bioremediation potential [109]. Functional activity of microbes during the composting of chlorophenol contaminated sawmill soil was studied, and it was observed that more than 90% of 1000 mg/kg of chlorophenols was removed in less than five months [111]. Another study of bioremediation of chlorophenol (30 mg/kg) from contaminated soil by indigenous microorganism was conducted where 90 % of 4–CP and 2,4,6-TCP was removed within 60 days and complete degradation of PCP was shown within 160 days [112]. A comparative study of chlorophenol degradation by two different inoculants, i.e. straw compost and remediated soil, by indigenous soil microbes, was done and results show that the contaminated soil with bark and without inoculums mineralized 63±0.4% of the [^{14}C] PCP, depicting high potential and activity of indigenous microorganism [113].

Table 2.3: Microorganisms present in the natural habitat capable of tolerating/utilizing chlorophenol compounds

Isolated Strain	Isolation Source	Congener	Optimum growth condition		Max Tolerance Conc. (mg/l)	Reference
			Temp (ºC)	pH		
Acinetobacter sp. ISTPCP-3	Sediment core of pulp and paper mill effluent discharge site at Nainital, India	PCP	30	7	100	[114]
B. cereus ITRC S6, *Serratia arcescens* ITRC S9	Pulp and paper mill effluent sludge, Nainital, India	PCP	30	7	300	[115]
S. chlorophenolica	PCP contaminated soils located in Southern Taiwan	PCP	30	7.2	100 200	[116]
*B. subtilis*OS1, *Alcaligenes sp.* OS2	Petroleum oil-contaminated soil located in Egypt	4-CP	30	-	100 100	[49]
Bacillus sp.	Chlorophenols contaminated soil from the industrial zone at Umm-SaiedCity, Qatar	2-CP	30	7	320	[117]
B. licheniformis	Rhizosphere soil in Phragmites wetland	4-CP	30	7	120	[118]
P. alcaligenes	Wastewater of pharmaceutical industries	24-DCP	35	7	380	[119]
*C. albicans*PDY-07	Activated sludge from the Luoyang Refinery Plant in Henan of China	4-CP	35	7	<440	[120]
*C. tropicalis*CTM-2	Acclimated activated sludge took from Tianjin Gasworks in China.	4-CP	30	7	400	[121]
*P. stutzeri*CL7	Secondary sludge of pulp and paper mill	PCP	37	7.5	600	[122]

P. mendocina NSYSU	PCP-contaminated soils	PCP	30	6	150	[123]
Bacillus consortium	Oil refinery plant waste sludge	24-DCP	28	6.5	407	[11]
Pseudomonas sp. SR3	Soils from former wood preserving facilities in central and north-western Florida	PCP	24		175	[124]
Pseudomonas sp. UG25 *and* UG30	Soil samples collected from the 0 to 30 cm layer of a PCP-contaminated industrial site in Ontario, Canada.	PCP	30	7.2	250	[125]
Pseudomonas sp. Stenotrophomonas sp.	Samples from the Riachuelo, a polluted Buenos Aires River	TCP	28	7	100	[126]
S. paucimobilis, Burkholderia cepacia, Chryseomonas luteola, Vibrio metschnikovii	Compiègne municipal waste treatment activated sludge (France)	246-TCP	28	7	200	[127]
Rhizobium sp. 4-CP-20	PCP contaminated soils located in Southern Taiwan	4-CP	30	7	100	[128]
A. chlorophenolicus A6	Aridic haplustoll, sandy loam	4-CP	23-28		350	[129]
Mixed microbiota	forest soil located in a coast-to-inland transect from central Chile	246-TCP			5000	[109]

2.6.2. Biodegradation of chlorophenols in the sediment

Biodegradation of various chlorophenols by indigenous microbes present in sediments and groundwater have been studied by many researchers. Degradation of six dichlorophenols isomers were investigated in the sediments from five ponds near Athens and also degradation of PCP, 2,4-D and 2,4,5-T were investigated using sediments collected throughout the united states and the soviet union [130]. Sediments from three different freshwater sites near Pensacola had shown ability to degrade 2-CP, 3-CP and 4-CP under methanogenic enrichments [131]. In the presence of sulfate, 2,4-DCP and 4-CP were transformed anaerobically to 4-CP and phenol respectively, in the freshwater lake sediments between 18-40 °C [132].

It was reported that complete oxidation of chlorophenols coupled to the reduction of nitrate, ferric iron, sulfate and other alternate electron acceptors, can be an energetically favorable process [133]. Three isomers of CP (2-, 3-, 4-CP) and DCP (2,5-, 3,4-, 3,5-DCP) and PCP were dechlorinated by a consortium in anoxic estuarine sediment slurries. The removal rate of PCP by unacclimated sediment was 0.16 mg/L/d and by adapted sediments was in the range of 0.27 to 0.66 mg/L/d [108]. PCP was dechlorinated via 2,3,4,5-TeCP, 3,4,5-TCP, and 3,4- or 3,5-DCP under sulphate reducing conditions where removal of 3,4,5-TCP was the rate limiting step. The sediment collected from Tsurumi River, Japan was studied for the anaerobic reductive transformation of halogenated aromatics under sulfidogenic conditions. Biodegradation rate for chlorophenols was reported in the range of 0.01 to 0.377 d^{-1}[134]. 2-CP, 3-CP, 4-CP, and 2,4-DCP were degraded anaerobically with a rate of 8 to 37 µmol/L/d under a sulfate-reducing condition with an estuarine sediment inoculums [74]. Haggblom et al. (1993) also examined the anaerobic biodegradability of 2-, 3-, 4-CP and 2-, 3-, 4-chlorobenzoate in the estuarine and freshwater sediments from the Hudson River and the East River, using nitrate, sulfate, and carbonate as electron acceptors[135]. Under denitrifying conditions, 3- and 4-chlorobenzoate was degraded with stoichiometric nitrate loss. Under sulfidogenic conditions, 3- and 4-chlorobenzoate, and all three monochlorophenol isomers were utilized, while under methanogenic conditions all compounds except 4-chlorobenzoate were metabolized.

Many groups also examined biodegradation of chlorophenol in groundwater. Heterotrophic bacterial communities, isolated from oligotrophic psychrophilic lakes without industrial or urban activity, had shown the ability to degrade both 2,4,6-tribromophenol and 2,4,6-trichlorophenol efficiently as a sole carbon and energy source [136]. In situ, chlorophenol bioremediation potential of the indigenous bacterial community from boreal groundwater was studied. Several gram-negative and gram-positive bacterial strains were isolated which shows a high potential for degradation of 2,3,4,6-TeCP and PCP [137]. Davis et al. (1994) studied the attenuation and biodegradation of chlorophenols in groundwater at a former wood treating facility. Results imply that at the plume periphery, at a concentration below 0.05 mg/L, PCP will absorb to the aquifer matrix and at a concentration below 20 mg/L, PCP biodegradation is apparent in the subsurface. While at higher concentration PCP is likely to be mobile and more recalcitrant to degradation [138].

2.7. Biotransformation of chlorophenols in Engineered System

Biodegradation of chlorophenols in the soil, effluent, waste water and sludge under a controlled environmental condition in the laboratory were reported at large. Both aerobic and anaerobic biodegradation processes have been studied in reactors under different physiological conditions.

2.7.1. Aerobic reactors

Table 2.4 summarizes the results of the biodegradation of chlorophenols in different aerobic bioreactors. Mainly two types of bioreactors have been effectively utilized for removal of toxic compounds. First is fixed bed bioreactors such as packed bed reactors (PBR) and second is particle based bioreactors such as fluidized bed reactors (FBR). Both types of bioreactors have their advantages and disadvantages. PBRs have shown higher loading rate of toxic compounds leading to higher removal rate compares to FBR. However mass and oxygen transfer limitation and clogging of packing bed can limit the efficiency of the PBR [21, 24]. Mixture of 2-CP, 4-CP, 2,4-DCP and 2,4,6-TCP (equal concentration of each CPs) were mineralized by 92.7% and 99.7% at 14 ºC and 23 ºC temperature respectively, in PBR with high volumetric loading rate of 1027.2 mg CPs/L reactor/d[24]. Use of net draft tube (internal or external) in which air is used for liquid circulation in fixed bed bioreactor can eliminate the problem of mass and oxygen transfer to some extent. A novel air-lift bioreactor, with a honeycomb-like ceramic column packed in the inner draft tube as the carrier for immobilization of microbial cells, was used for biodegradation of 2,4-DCP and phenol with removal efficiency of above 88% [21]. In one such PB-ALR with high A_D/A_R ratio, reactor's downcomer was packed with a porous support of volcanic stone fragments [22]. These PB-ALR configuration supports higher oxygen mass transfer, low shear stress and high comparable mixing time than that obtained with the unpacked reactor. Also, the erosion of biofilm could reduce by liquid velocities pattern created by radial flow through the porous support since the higher shear stress occurs into the riser [22, 100]. Several studies have also been conducted with fluidized bed reactors (FBR) for mineralization of various chlorophenols as mentioned in Table 2.4 [139, 140]. Compare to PBRs, in FBRs and ALRs problem of clogging and mass and oxygen transfer limitation can be prevented, but it has a disadvantage of washout and detachment of biomass at high flow rate. The immobilization of microbes or a biofilm reactor has an advantage of high cell density and high specific biofilm surface which leads to high volumetric removal of toxic compounds [22, 24, 139]. Different varieties of supports such as volcanite, granular activated carbon, diatomaceous earth, ceramic filters, polyvinyl alcohol, Na-alginate, Agarose, glass beads, etc. are reported for biofilm formation and immobilization [21]. Immobilization can provide certain advantage such as prevent loss of biomass, reutilization of biomass and resistant to high toxicity.

There are certain factors that affect the removal efficiency of bioreactors such as hydraulic retention time (HRT), dissolved oxygen concentration, recirculation rate, air flow rate and substrate loading rate, etc. Eker and Kargi (2007) studied the effect of HRT (5 to 30

h) on removal of 2,4,6-TCP at constant concentration of 300 mg/L by in hybrid loop reactor consisting PBR and an aeration tank with recirculation[141]. It was observed that toxicity and TCP removal rate increased with increasing HRT. In another same study effect of initial 2,4,6-TCP concentration (50-450 mg/L) on removal rate was studied at constant HRT and SRT and reported that at higher substrate concentration removal efficiency decreases [142]. Melin et al. (1997) also reported the same observation that at lower HRT the effluent concentration of PCP increases and at certain point system become unstable and breakdowns [140].

Membrane bioreactor (MBR) technology is also important for treatment of toxic effluent. MBR has an advantage that it gives good quality effluent, low sludge production, higher loading rate, and capable of degrading a wider range of toxic compounds. However, membrane fouling and extra energy requirement leads to higher operating cost [143]. MBR was successfully employed to treat PCP contaminated wastewater with a loading rate of 240 mg PCP/L reactor/d [143].

Bioreactor system has also been applied for degradation of chlorophenols using fungi and yeast. Galíndez-Mayer et al. (2008) reported 98% degradation of 4-CP by yeast *Candida tropicalis* in FBR with a loading rate of 4.1 mg 4-CP/L/h [139]. Three species of white rot fungi *Trametes versicolor*, *Phanerochaete chrysosporium* and *lnonotus dryophilus* have shown dehalogenation of PCP in rotating tube reactor with a removal rate of 0.1 mg/L/d [144]. Other species of fungi such as *Panustigrinus*[145], *Trametes versicolor*[146], *Bjerkandera adusta* and *Anthracophyllum discolor* [58], *Phanerochaete chrysosporium* [147] have been shown to degrade various chlorophenols in different bioreactor systems.

2.7.2. Anaerobic Reactors

Table 2.5 summarizes the result of the biodegradation of chlorophenols in different anaerobic reactors. Anaerobic bioreactors such as up-flow anaerobic sludge blanket (UASB) and fixed film reactors also employs biofilm technology for enhanced bioremediation efficiency. Biofilm provides high volumetric biomass and resistance to toxic compounds which improves the bioreactor stability under adverse operating conditions [71, 148]. UASB is an anaerobic reactor system in which an anaerobic granular sludge with high metabolic activity is used for the treatment of toxic compounds in wastewater. It is a simple, economically feasible and efficient treatment process with high biosolids content and operates at low hydraulic retention time. UASB reactors have been successfully utilized by many researcher for biodegradation of lower to higher chlorophenols with loading rate ranging from 80 mg CP/L reactor/d to 226 mg CP/L reactor/d and removal efficiency of 70 to 100%[23, 92, 149-152]. UASB reactor augmented with *Desulfitobacterium frappieri*PCP-1 was assessed for the degradation of PCP. A PCP removal efficiency of 99% and a dechlorination efficiency of not less than 90.5% were observed throughout the experiment, with 3-CP and phenol being observable dechlorination intermediate[153]. Complex mixture of chlorophenols, commonly found in environmental condition, including 2-CP, 4-CP, 2,4-DCP, 2,4,6-TCP and PCP were efficiently treated using UASB reactor at loading rate of 182 mg CPs/ L reactor/d and with removal rate of nearly 70.7% [149].

Other bioreactors like Horizontal-flow anaerobic immobilized biomass (HAIB) reactor and FBR have also been utilized by some investigators for mineralization of PCP at a lower concentration. PCP loading rate up to 5.2 to 10.67 mg PCP/L reactor/d was observed in HAIB with nearly 100% removal efficiency [148, 154-157]. In some studies PAM (partially aerated methanogenic) condition was also tested for complete removal of chlorophenol compounds. PAM process could provide more diverse catabolic pathways for achieving complete removal of toxic compounds and its metabolites [157].

An expanded granular sludge bed reactor has been investigated for the anaerobic treatment of wastewater bearing PCP at psychro-mesophilic condition (17-28 ^{o}C). PCP with a loading rate of up to 16 mg PCP/L reactor/d was efficiently treated with complete removal and methanization efficiencies higher than 50% observed [158]. Lower concentration of PCP with 2.534 mg PCP/L reactor/d loading rate was degraded in packed bed column with 99% degradation. PCP was dechlorinated to phenol with a small quantity of 3-CP as an intermediate showing meta-dechlorination [159].

Lower chlorophenols have also been treated by anaerobic reactors such as 2-CP in anaerobic fixed bed reactor (AFBR) [160], 4-CP in UASB [150], and 2,4-DCP in UASB [23] with observed removal efficiency of 70 to 88%. Biodegradation efficiency for lower chlorophenols was observed to be diminished under anaerobic condition. Co-metabolism or biodegradation of higher chlorophenols in the presence of lower chlorophenols or microbial easily degradable substrate such as glucose, sucrose, peptone, yeast extract etc has found to increase the removal efficiency and dechlorination rate under both aerobic and anaerobic condition [16, 149, 151].

2.7.3. Sequencing anaerobic/aerobic reactors

Sequencing anaerobic/aerobic reactors have been also studied by many researchers for complete degradation of chlorophenols andtreatment of wastewater. It’s been reported that the rate of dechlorination by anaerobes for multiple chlorinated phenols decreases with dechlorination process resulting in the accumulation of intermediate low chlorinated phenols. Aerobic biodegradation process could subsequently degrade These low chlorinated phenols generated as by-product from anaerobic process. Thus,combination of the anaerobic and aerobic process is important for complete mineralization of chlorophenols compounds in the wastewater [160, 161]. Chlorophenol containing wastewater was sequentially treated by a combination of upflow anaerobic sludge blanket (UASB) and aerobic rotating biological contactor (RBC) having higher biomass concentration and higher sludge retention time (SRT). 30 mg/L of each 2-CP and 2,4-DCP was efficiently treated with an effluent concentration below 0.1 mg/L[162]. The study of the degradation of 15 mg/L of PCP by anaerobic and combined anaerobic-aerobic process resulted in 93% and 97% removal efficiency respectively [163]. 2,4,6-TCP was successfully and completely degraded in a two-stage anaerobic-aerobic biological process in which initially 2,4,6-TCP was transformed into 2,4-DCP and then 4-CP anaerobically by reductive dechlorination which was further attacked and completely degraded aerobically in a second stage [161]. Sequential anaerobic-aerobic GAC-FBR has also been used for the treatment of PCP. The first PCP is converted to mono

chlorophenol and phenol anaerobically which are further completely degraded by aerobic GAC-FBR [164].

Table 2.4: Biodegradation of chlorophenols in the aerobic reactor

Reactor	Congener	Process parameter	Description	Temp. (°C)	V (mg/L/d)	η (%)	Reference
PBBR	246-TCP Comet	A_D/A_R = 10.7 H_{PB}/D_C = 1.22 HRT = 2.95 h Q_{AIR} = 8 L/min U_G = 5.61 cm/s	Net draft tube riser for aeration and liquid circulation used; Tezontle (volcanic stone) D> 3 mm as packing material; *Achromobacter* sp.		46.88	99.9	[22]
ALR	24-DCP Phenol Comet	A_D/A_R = 3.24 HRT = 8 h Q_{AIR} = 8.33 L/min	Ceramic honeycomb carrier with 5*5 mm square hole; *Achromobacter* sp.		85.5	97.8	[100]
ALR	24-DCP Phenol Comet	A_D/A_R = 3.24 HRT = 6.25 h Q_{AIR} = 8.33 L/min	Ceramic honeycomb carrier with 5*5 mm square hole; *Achromobacter* sp.		26 - 394	100 - 88	[21]
PBR	2CP 4-CP 24-DCP 246-TCP Growth	H/D = 4.9 Q_{AIR} = 0.09 L/min HRT = 2.5 h Q_{LIQ} = 2.4 L/h Recirculation rate = 0.041 L/min	Foam Glass Bead (5-7 mm); Mixed Bacterial Consortium; Two bioreactors RA and RB at 14 and 23 °C respectively.	23 14	1027.2 (Equal conc. of each CP)	99.7[a] 92.7[b]	[24]
PAM-FBBR	246-TCP Phenol ± Sucrose Comet	H/D = 41 Q_{AIR} = 2 vvd HRT = 24 h	Bed of bioparticles consisted of anaerobic microorganisms (aggregated into granules) and a selected Mixed aerobic community immobilized in k-carrageenan.	35	120 (TCP) 30 (Phe) 1000 (COD-Sucrose)	99.9 99 97	[157]
HLBR system	246-TCP COD (Molasses)	H/D = 5.7 Q_{AIR} = 5 vvm HRT = 25 h SRT = 20 d Q_{LIQ} = 0.092 L/h	Consisted of packed column biofilm and an aerated tank bioreactor with effluent recycle; olive pits with particle size of 1 cm and volume of 0.5 cm^3 used as biocarrier. Activated sludge.	23±2	48 – 375 2400	90 90	[142]

FBR	PCP	H/D = 12.75 HRT = 5 h	Porous Celite R-633 diatomaceous earth as Bio –carrier. Activated sludge.	26	13.6	99.9	[140]
FBR	4-CP Phenol	H/D = 7.27 U_G = 0.57 to 1.72 cm/s. HRT = 24.4 h Q_{LIQ} = 52 mL/h	*C. tropicalis* CC1Yeast immobilization on Granular activated carbon	28	97.5 1378.2	98.7	[139]

V = volumetric loading rate (mg/L/d); η = removal efficiency (%); QAIR= ml/min; UG= superficial gas velocity (cm/s); QLIQ = superficial upflow velocity; vvd: volumes of air per volume of reactor per day; vvm = L air/L liq. Min; VR= working volume of reactor; HBP = height of packed bed; Dc= column diameter; Comet: Co-metabolism; Growth: Sole carbon and energy source; NS = not stated; HLBR: hybrid loop bioreactor system; STR = stirred tank reactor; FBR: fluidized bed reactor; PBR: packed bed reactor; ALR: Airlift reactor; PAM-FBBR: partially aerated methanogenic - fluidized bed bioreactor; PBBR; packed bed biofilm reactor

Table 2.5: Biodegradation of chlorophenols in the anaerobic reactor

Reactor	Congener	Process parameter	Description	Temp. (ºC)	V (mg/L/d)	η (%)	Reference
UASB	24-DCP Glucose	HRT= 13.2 h	Anaerobic micro flora	30±3	226	70.4	[23]
UASB	2-CP, 4-CP, 2,4-DCP, 246-TCP, PCP	HRT= 13.2 h	Acclimated sludge	30±3	182 (Total CPs)	70.7	[149]
UASB	PCP Comet	HRT = 21.6 h	Anaerobic sludge acclimated to PCP	28	201 (PCP) 6189 (COD)	100 97.4	[151]
UASB	PCP Comet	HRT = 20-22 h	Anaerobic sludge acclimated to PCP. Studied the effect of microbial easily degradable substrate (MEDS).	28	120 (PCP) 1375 (Sucrose)	99.8 90	[92]

UASB	PCP Comet	HRT = 20-22 h	Anaerobic sludge acclimated to PCP.	28	204	100	[152]
FFR	PCP Comet	H/D = 12.36 HRT = 16.8	Small stone (5-10 mm). Anaerobic sludge.		60	99	[71]
FBR	PCP Lactate Comet	HRT = 24 h H/D = 8.75	Celite R-633 (0.3-0.6 mm); Acclimated sludge.	35	5	NS	[156]
PBR	PCP	H/D = 2.88 HRT = 182.4 Q_{LIQ} = 0.01 mL/min	Glass bead (2 mm) and Soil slurry.		2.534	99	[159]
HAIB	PCP Comet	L/D = 20 HRT = 18 h	Polyurethane foam cubes of 0.3 ± 0.1 cm size; Acclimated sludge	30±3	4.2 (PCP) 1700 (COD)	99 99	[155]
HAIB	PCP Glucose Comet	L/D = 20 HRT = 18 h	Polyurethane foam cubes of 0.3 ± 0.1 cm size; Acclimated sludge	30±2	11.3	100	[148]
AFBR	2-CP Growth	HRT = 81.6 h	Packed with clay bead with a diameter of 12 ± 1 mm. Acclimated sludge	37±2	758	80	[160]

UASB: Up-flowanaerobic sludge blanket; HAIB: Horizontal-flow anaerobic immobilized biomass reactor; FFR: fixed film reactor; AFBR: anaerobic fixed bed reactor.

2.8.Kinetic study of chlorophenol degradation

Determination of biokinetic parameters is important for understanding the mechanism of bioreaction, for design and operation of the bioreactor and to define the optimal conditions for the removal of chlorophenol in biological treatment systems.

Table 2.6 and 2.7 summarize the kinetic parameters of chlorophenol biodegradation in aerobic and anaerobic environment respectively. Since the chlorophenols are toxic compound and have an inhibitory effect on microorganism at higher concentration, the substrate inhibition kinetic models are mostly used to describe the growth kinetic in the presence of chlorophenols. The different models derived for depicting growth inhibition kinetics has been summarize in Table 2.8 [165-167].

The inhibition constant (*Ki*) is an important parameter which shows the inhibition effect of the toxic compound. Higher the *Ki* value, lower the inhibition effect of substrate on the microorganism. For aerobic biodegradation of chlorophenols, the inhibition constant reported was mostly in the range of 1.5 to 354 mg/L. For a lower concentration of toxic compounds where inhibition effect is negligible, the growth kinetic follows the Monod kinetic model as mentioned in Table 2.8. For aerobic organisms, cell yield observed was in the range of 0.143 to 0.412 g dwt biomass/g chlorophenol for mono- and tri-chlorophenol, while for tetra- and penta-chlorophenol its range was from 0.062 to 0.4 g dwt biomass/g chlorophenol. Cell yields observed for anaerobic degradation was low and valued from 0.016 to 0.043 g dwt biomass/g chlorophenol. Low biomass yield in case of anaerobic biodegradation might be due to low energy yield from the metabolism as compared to aerobic biodegradation. While, in case of the aerobic process, the cell yield remains comparatively higher leading to the higher specific activity of substrate utilization. Relatively high cell yield, 7.47 g dwt biomass/g PCP, was observed for dechlorination of PCP by the mixed microbial consortium [159]. Biodegradation rate reported for anaerobic culture lies in between 0.018 to 8.9 mg Chlorophenol/mg biomass/h with most values below one. An exceptionally high activity of 7456 mg Chlorophenol/mg biomass/h was reported for 2-CP biodegradation by induced culture under anaerobic condition. The strain *Anaeromyxobacter dehalogenans* 2CP-C was induced by phenol, 2-chlorophenol, 2,4-dichlorophenol, 2,5-dichlorophenol, 2,6-dichlorophenol, and 2-bromophenol in which 2-bromophenol induced culture has shown highest dechlorination activity [86]. In case of aerobic culture, biodegradation rate was in the range of 0.036 to 358.4 mg Chlorophenol mg^{-1} biomass hr^{-1}. It is noted that specific activity decreases with increase in chlorination during aerobic degradation because chloride molecule can sterically hinder the action of monooxygenase enzymes, which normally initiate the degradation of aromatic rings [10]. Therefore, the aerobic system is more suited for biodegradation of less chlorinated phenol such as mono and di-chlorophenols while the anaerobic system is more suited to reductive dechlorination for highly chlorinated phenols [21, 92, 151, 152]. Half saturation coefficient (*Ks*) had a value between 0.01 to 0.96 mg/L for anaerobic degradation and 0.016 to 427 mg/L for aerobic degradation with most of the values below 50 mg/L. The low value of half saturation coefficient shows high affinity of the organism for chlorophenol. The maximum specific

growth rate is low with value in the range of 0.009 to 0.3 hr^{-1} for aerobic degradation while in case of anaerobic degradation it has a value ranging from 0.058 to 10.17 hr^{-1}. The specific growth rate is high for low concentration of chlorophenol and decreases with increase in chlorophenol concentration as it is inhibitory to the microorganism. In case of mixed microbial consortium and organisms which are previously enriched and acclimatized to the low concentration of chlorophenols has the high affinity and specific activity [140, 156, 159, 168].

2.9. Optimization of experimental parameters

Chlorophenols are toxic compounds and have inhibition effect on the growth of microorganism and thus limit the degradation capacity of the microorganism. This inhibition effect can be diminished to some extent by optimizing the experimental parameters. Different experimental conditions such as pH, temperature, nutrient, substrate concentration, etc. affects the degradation capability of an organism by altering it's the growth and physical properties of the compound i.e. 2,4-DCP [10, 165]. The optimization of these culture conditions can significantly improve the degradation efficiency. A specific range of pH value requires for maximum growth of each microorganism, extreme pH value i.e. acidic or alkaline condition is inhibitory to the growth of bacteria. During the 2,4-DCP the value of pH drops due to the release of chloride ion which affects the growth and degradation capacity of bacteria [114, 169]. Temperature affects the growth and activity of microorganism. At higher temperature, enzymes lose their structure and reduce the degradation efficiency. Also at the low temperature, bacterial activity decreases which increases the inhibition effects of toxic compounds. The optimum temperature range mostly used for biodegradation by the microorganism is 25 to 35 ºC [114, 122]. Inoculum size is another factor that affects the overall specific degradation rate. The specific degradation rate increases with increasing inoculum size as the total biomass or microorganism increases in the medium. As the biomass increases in the medium, the degradation rate per cell volume increases. However, it was reported that the after certain inoculum size, an increase in inoculum size does not have a significant effect on growth and biodegradation due to limitation nutrient condition [170, 171]. The addition of different carbon and a nitrogen source such as glucose, $(NH_4)_2SO_4$, peptone, yeast extract, etc. also alters the degradation capacity of the microorganism [165, 172, 173]. Nitrogen source is important for bacterial growth and expression of different enzyme systems. The optimum concentration of nitrogen source can increase the growth of microorganism and enzyme expression. Thus, optimization of experimental conditions such as pH, temperature, nitrogen source and inoculum size can improve the degradation efficiency of the microorganism.

Optimization with conventional method i.e. single parameter at once is time-consuming and less economical. However, optimization by using a statistical method such as response surface methodology is more suitable as it simultaneously optimizes the various parameters and their interaction effects at different levels. Response surface methodology (RSM) is an important statistical and mathematical design used for determining the influence of different factors on desired response and optimizing the desired response [174]. The RSM is more economical and time saving than conventional techniques as the minimum number of

experiments require for getting the desired optimum response. Several studies have been reported that used the RSM for the optimization process. In one such study, the effects of environmental parameters, i.e, pH, temperature, time and enzyme concentration were evaluated on biodegradation of 2,4-DCP with laccase from *Pleurotus* sp. using Box-Behnken design of experiment and the maximum 98% degradation of 2,4-DCP was achieved [175]. In another study, the central composite design of RSM was used for optimizing the multiple responses, i.e. maximum 4-CP biodegradation, and specific growth rate. The 4-CP biodegradation efficiency was found 23% higher at RSM optimized conditions than that obtained at un-optimized culture conditions [169]. A 5-fold enhancement in secondary carotenoid lutein production by the green microalgae *Auxenochlorella protothecoides* SAG 211-7a was achieved using the central composite rotatable design of RSM [176]. The box-behnken design of experiment was used for optimization of the enzymatic conversion of widely available lignocellulosic biomass-wheat straw (WS) and the combined effects of enzyme loading, substrate concentration, surfactant concentration, and reaction time on hydrolysis yield from enzymatic saccharification of WS were successfully studied [177].

In the present study, the central composite design of RSM was used for optimization of experimental parameters. The total number of experimental runs was: $n = 2^k + 2k + n_0 = 31$, where k (=4) is the independent variables and n_0 (=7) is the number of centre points used in the design. The factor levels are coded as -1 (low), 0 (central point), and +1 (high). The relationship between the coded and actual value is described by Equation 2.1.

$$X_i = \frac{U_i - U_0}{\Delta U} \qquad \text{(Eq. 2.1)}$$

Where, X_i is the coded level of the independent variable, U_i is the actual level of the independent variable, U_0 is the uncoded level of the independent variable at its centre point and ΔU is the step change value.The default value of α =2 was taken in the experiment.

The second order polynomial regression model used for the fitting the experimental data by response surface method is defined Equation 2.2.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \qquad \text{(Eq. 2.2)}$$

Where, Y= predicted response, k = number factor variables, X_i&X_j= independent variables. The second order regression model is significant for optimization process as it count the interaction effect between variables and surface curvature. The adequacy of the fitted regression model is checked by using the analysis of variance (ANOVA) and regression analysis. The robustness of the model can be checked with the help of coefficient of determination (R^2-value) and p-value. All the statistical analysis was performed using the Minitab v16 (trial version), USA.

Table 2.6: Kinetic parameters for aerobic degradation of chlorophenols

Congener	Microorganism	Ks (mg/L)	Ki (mg/L)	μ_m (1/h)	Activity (mg S/ gVSS/ h)	Cell yield, (g dwt/g)	Reference
2-CP	*Aerobic granules*	24.61	315.02		35		[178]
2-CP	*R. erythropolis* M1		354.28	0.248			[179]
3-CP	*Acclimated sludge*	47.32	30.54	0.009	25	0.412	[180]
4-CP	*A. chlorophenolicus* A6	30.83	275	0.22			[169]
4-CP	*C. tropicalis* CTM-2	1064.6	5.10	5.28			[121]
4-CP	*R. erythropolis* M1		164.67	0.0494			[179]
4-CP	*Acclimated sludge*	34.98	13.77		130.3		[181]
4-CP	Mixed microbial consortium	1.104	194.4		92		[16]
4-CP	*Acinetobacter sp.*				150[b]		[96]
4-CP	Activated sludge	71			117.1		[182]
4-CP	Aerobic granules	44.8	74.5		61		[183]
24-DCP	Aerobic granules	175.2	4.6		358.4		[183]
24-DCP	*P. putida* CP1	427	1330				[184]
24-DCP	*R. erythropolis* M1		81.34	0.0244			[179]
24-DCP	*Flavobacterium sp.*	11.5	12.5	0.3			[185]
24-DCP	*Acclimated sludge*	79.74	44.46		112.4		[181]
26-DCP	*Sphingomonas sp.* P5					0.210	[186]
246-TCP	Aerobic granules	64	39.2		14		[183]
236-TCP	*Sphingomonas sp.* P5					0.158	[186]
246-TCP	*R. rhodochrous* (DSMZ 43241)	73.55	338		2.69[b]		[187]
246-TCP	Microbial consortium		610	0.01	34[a]	0.17	[127]
246-TCP	Microbial consortium				33.96		[2]

246-TCP	*Sphingomonas sp.* P5					0.150	[186]
246-TCP	*P. aeruginosa* *Achromobacter sp.*	0.477		0.093		0.143	[18]
246-TCP	*Acclimated sludge*	4.2			27.12		[188]
246-TCP	*Arthrobacter sp.*			0.13		0.26	[189]
2346-TeCP	*Acclimated sludge*	5.5			23.63		[188]
2346-TeCP	*Sphingomonas sp.* P5					0.092	[186]
2346-TeCP	*Nocardioides sp.* K44	0.46	5.14	0.19			[190]
2346-TeCP	*Sphingomonas sp.* K74	2.4	0	4.9			[190]
2356-TeCP	*P. aeruginosa* *Achromobacter sp.*	0.71		0.052		0.063	[18]
PCP	Acclimated Mixed Bacteria	0.06	1.375	0.074		0.136	[168]
PCP	Activated sludge	0.016	5.3		8.54	0.096	[140]
PCP	*M. chlorophenolicus*	0.885			0.036	0.4	[191]
PCP	*Arthrobacter sp.* ATCC 33790	1.12		0.154		0.16	[192]
PCP	*Pseudomonas Cepacia*		222				[193]
PCP	*Flavobacterium sp.* ATCC 39723	25	1.5	0.3		0.12	[194]
PCP	*Flavobacterium sp.*	38	81	0.3			[185]
PCP	*Sphingomonas sp.* P5			0.142		0.076	[195]
PCP	*Sphingomonas sp.* P5					0.062	[186]
PCP	*P. aeruginosa* *Achromobacter sp.*	0.589		0.062		0.079	[18]
PCP	*Acclimated sludge*	0.6			1.23		[188]
PCP	*Arthrobacter sp.*			0.1		0.15	[189]

[a] – assume 1 mg cell = 0.5 mg Protein; [b] – activity mg S/ g dwt/h

Table 2.7: Kinetic parameters for anaerobic degradation of chlorophenols

Congener	Microorganism	Ks (mg/L)	Ki (mg/L)	μ_m (1/h)	Activity (mg S/ gVSS/h)	Cell Yield (g dwt/g)	Reference
2-CP	Activated sludge	0.67		2.34	0.98		[149]
2-CP	*A. myxobacter* (non-induced)				8.9[a]		[86]
2-CP	*A. myxobacter* (induced)				7456[a]		[86]
2-CP	*Desulfovibrio dechloracetivorans sp.*					0.016	[85]
2-CP	Acclimated biofilm				0.29		[29]
2-CP	Acclimated sludge					0.024	[196]
2-CP	Strain 2CP-1					0.047	[197]
3-CBA	Acclimated sludge					0.043	[198]
4-CP	Acclimated biofilm				0.3		[29]
4-CP	Activated sludge	4.5		2.95	0.49		[149]
4-CP	*C. albicans* PDY-07	0.87	456.3	0.044			[120]
24-DCP	Acclimated biofilm				0.25		[29]
24-DCP	Activated sludge	4.88		10.16	0.67		[23]
24-DCP	Activated sludge	4.88		10.17	0.635		[149]
24-DCP	Activated sludge	0.05					[156]
26-DCP	*Desulfovibrio dechloracetivorans sp.*					0.033	[85]
35-DCP	Activated sludge	0.58			0.018		[199]
235-TCP	Activated sludge	0.58			0.053		[199]
245-TCP	Activated sludge	0.16					[156]
246-TCP	Activated sludge	0.96		2.83	0.955		[149]

246-TCP	Acclimated biofilm			0.24		[29]
246-TCP	Activated sludge	0.02				[156]
345-TCP	Activated sludge	0.05				[156]
2345-TeCP	Activated sludge	0.09				[156]
2346-TeCP	Activated sludge	0.01				[156]
2356-TeCP	Activated sludge	0.58		0.034		[199]
PCP	Activated sludge	0.11				[156]
PCP	Activated sludge	0.67	2.75	0.525		[149]
PCP	Activated sludge	0.58		0.019		[199]
PCP	Mixed consortium	0.08	0.058		7.47	[159]
PCP	Acclimated sludge			0.42		[152]
PCP	Desulfomonile tiedjei DCB-1			1.43[a,b]		[81]
PCP	Acclimated sludge			0.36		[92]
PCP	Acclimated sludge			0.40		[200]
PCP	Acclimated sludge			0.15		[201]
PCP	Acclimated sludge			0.41		[202]

3-CBA= 3-chlorobenzoic acid; [a] - activity mg S/g dwt/h; [b] - assume 1 mg cell = 0.5 mg Protein

Table 2.8: Different kinetic models reported for kinetic study of chlorophenolic compounds.

Authors	Model	References
Monod	$\mu = \frac{\mu_m S}{S + K_s}$	[203]
Andrews	$\mu = \frac{\mu_m\, s}{s + k_s + \frac{s^2}{k_i}}$	[167]
Yano	$\mu = \frac{\mu_m\, S}{S + K_s + \frac{S^2}{K_i}\left(1 + \frac{S}{K}\right)}$	[204]
Edwards	$\mu = \mu_m \left[\exp\left(\frac{-S}{K_i}\right) - \exp\left(\frac{-S}{K_s}\right)\right]$	[166]
Webb	$\mu = \frac{\mu_m\, s\, \left(1 + \frac{S}{K}\right)}{s + k_s + \frac{s^2}{k_i}}$	[205]
Aiba	$\mu = \frac{\mu_m S \left[exp\ \frac{-S}{K_i}\right]}{S + K_s}$	[206]
Multisubstrate Monod Kinetic Model	$\frac{\partial X}{\partial t} = \mu_T\, X = \left(\sum_{i=1}^{n} \mu_i\right) X$ $\frac{\partial S_i}{\partial t} = \frac{1}{Y_{X/S_i}} \cdot \frac{\mu_{m_i} S_i}{K_{S_i} + \sum_{j=1}^{n} \frac{K_{S_i}}{K_{S_j}} S_j} \cdot X$	[18]

μ- specific growth rate, μ_{max}- maximum specific growth rate, K_s- half saturation constant, K_i- substrate inhibition constant, K- Yano constant, S- Substrate concentration, X- Biomass concentration.

CHAPTER – III

MATERIALS AND METHODS

Chapter – III

MATERIALS AND METHODS

3.1 Sample collection

The soil, sludge, and effluent samples were collected from two different sites. First from the Rourkela steel plant, Rourkela, Orissa, India, and the second from the dye industries effluent treatment plant, Ahmedabad, Gujarat, India. All the samples were collected in an airtight container and stored at 4 ºC until further use.

3.2 Enrichment and isolation

For enrichment of the microorganisms, 10 gm of sludge and soil sample were added to 50 mL mineral salt medium (MSM) seperately with 0.5 g/L peptone and 2,4-DCP (20-200 mg/L) as sole carbon and energy source and incubated at 30 ºC, 120 rpm for 4-6 months (Figure 3.1). After every 15 days, 50 mL of enriched sample was centrifuged at 10,000 rpm for 10 min and the cell pallet was resuspended in the fresh MSM medium with increasing concentration of 2,4-DCP. The removal of 2,4-DCP was checked periodically. Isolation of pure bacterial strains from the final acclimated culture was done by using serial dilution technique and repeated streaking on MSM agar plates containing 1.5% agar and 50 mg/L 2,4-DCP.

3.3 Chemical and reagents

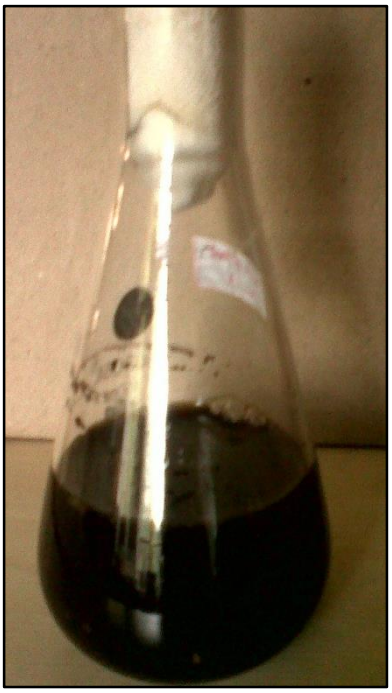
2-CP, 3-CP, 4-CP, 2,4-DCP and catechol (purity 97%) were of analytical grade and obtained from the Loba Chemie, India. 2,4,6-TCP and PCP (purity 97%) were supplied by Merck, Germany and Sigma-Aldrich, India respectively. The stock solution of all the chlorophenol compounds prepared in 0.02 M NaOH and the 1M orthophosphoric acid was used to adjust pH to 7.4±0.2. All the other inorganic chemicals used in the experiments were of analytical grade and obtained from Merck, India. HPLC grade methanol, acetic acid and hydrochloric acid supplied byHi-media, India was used for HPLC analysis.

3.4 Analytical method

Biomass concentration was determined by measuring optical density at 600 nm by UV-Visible spectrophotometer (Shimadzu UV-1800, Japan) [207].

The residual concentration of 2-CP, 3-CP, 4-CP, 2,4-DCP, 2,4,6-TCP and PCP were determined by HPLC system (Jasco, US) coupled with MD-2015 photodiode array detector and 2080 plus isocratic pump. The 1 mL sample was centrifuged at 10,000 rpm for 12 min and supernatant was filtered through 0.22 µm filter before analysis. The sample was acidified to pH 2with 1M hydrochloric acid before analysis. The column used was Agilent TC-C18 (25mm * 4.6 mm);sample was eluted at flow rate of 0.75 mL/min with mobile phase consisting of methanol: water (80:20);detection wavelength was set at 280 nm.

a



b

c

Figure 3.1: Enrichment and acclimation of different collected samples (a) Sludge collected from dye industries (b) Soil and effluent collected from dye industries treatment plant (c) Sludge, soil and effluent from Rourkela steel plant.

In the second approach for HPLC analysis of some chlorophenols, the sample was directly injected without acidification. The mobile phase consist of methanol: water: acetic acid (60:38:2);detection wavelength was set at 280 nm [122, 181].

2-CP, 3-CP, 4-CP, 2,4-DCP, 2,4,6-TCP, and PCP were also analyzed by UV-Visible spectrophotometer (Shimadzu UV-1800, Japan) at 273, 273, 279, 284, 310 and 318 nm wavelengths respectively [127, 208].

Electrospray ionization (ESI) mass spectroscopy (Perkin Elmer, Flexar SQ300 MS Detector) was used for identification of dichlorophenol and their biodegradation products. The analysis was carried out in split less mode; range of mass scan 50–400; with temperatures as follows: injector 260 ^{o}C, detector 280 ^{o}C; gradient program: 65 ^{o}C to 96 ^{o}C (4 ^{o}C min^{-1}), 96 ^{o}C to 160 ^{o}C (8 ^{o}C min^{-1}) and up to 230 ^{o}C (12 ^{o}C min^{-1}). The MS was equipped with a HP1 column of 30 m length, 0.25 mm internal diameter and 0.25 µm of film thickness. Helium was used as the carrier gas at a flow rate of 2.9 ml min^{-1}[11].

The chloride ion was quantitatively analyzed by silver chloride method according to the method of freier [209]. The sample aliquots (0.4 mL) were treated with 60 µL of nitric acid and then 50 µL of $AgNO_3$ added to the reaction mixture. After 10 min incubation in the dark, the absorbance was measured at 546 nm.

The concentration of chloride ions in the samples was also analyzed colorimetrically by EPA 9215 method [210]. 0.5 mL culture supernatant was mixed with 0.5 mL colorreagent, and mixture is kept for 10 min for color development. Color reagent is prepared by equally mixing solution A (saturated solution of mercuric thiocyanate in methanol) and solution B (20.2% ferric nitrate solution in 9 M nitric acid). The absorbance was measured by UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) at 460 nm. The chloride ion as quantified against the standard curve of sodium chloride (5-200 mg/L) [115, 210].

The presence of 5-chloro-2-hydroxymuconic semialdehyde (5-CHMS) was determined at 380 nm by UV-Visible spectrophotometer (Shimadzu UV-1800, Japan) [40].

Catechol and chlorocatecholwereanalyzed using the method of Arnow [211]. The samples were centrifuged at 10,000 rpm for 10 min to remove cells. The supernatant (0.5 mL) was treated with 0.5 N HCl (0.5 mL). After mixing, 0.5 mL of nitrite molybdate reagent was added to it and mixed resulting in a yellow color. Nitrite molybdate reagent was prepared by dissolving 10 gm of sodium nitrite and 10 gm of sodium molybdate in 100 mL of distilled water. After mixing, 0.5 mL of 1 N NaOH was added resulting in red color. Following the mixing, absorbance was measured at 510 nm.

3.5 Morphological and biochemical analysis

Morphology of the microbial cell was examined using Filed emission scanning electron microscopy (FE-SEM) and phase contrast microscopy. The pure bacterial culture was transferred onto glass slide with the help of loop and spread on it with drop of sterile distilled water under aseptic condition. The microbes were fixed to slide using 2% glutaraldehyde for 2 h. After fixation, the cells were washed/dehydrated with series of ethanol (30%, 50%, 70% and 90%) for 10 min at each concentration. Afterward, the cells were washed three times of 100% ethanol for 10 min each. After drying, the microbial cells were analyzed by FESEM and Phase contrast microscopy.

Biochemical analysis was done as per Bergey’s manual of determinative bacteriology [212]. Following are the details of the biochemical tests performed.

Methyl Red-Voges Proskauer: MR-VP broth medium was inoculated with the isolates and incubated at 30 °C for 24 to 48 h. after incubation, 2/3rd culture was transferred to new tube and added 0.2 mL methyl red indicator. A production of red color is positive test. The remaining 1/3rd culture was tested for Voges-Proskauer test. 0.6 mL of Barritt's solution A and 0.2 mL of solutioin B were added to the cultures and shake vigorously. Positive reaction was indicated by presence of red color after 15 minutes.

Starch hydrolysis: The isolates were inoculated onto the starch agar plates in a straight line and incubated at 30 °C for 24 to 48 h. after incubation period, several drops of Grams iodine were placed around the growth and allowed to stand for 15 minutes and observed for clear zone around the growth line. Clear zone around the colony indicates the hydrolysis of starch (positive test) and if no clear zone observed and medium turns blue indicates starch has not been hydrolyzed (negative test).

Casein hydrolysis: This test is performed to detect the presence of proteolytic enzymes. Skim milk agar plates were inoculated by streaking the isolates along the surface and incubated at 30 °C for 24 to 48 h. after incubation period; the plates were examined for clear zones around the colony against black background.

Gelatin hydrolysis: Gelatin agar tubes were inoculated with the isolates by stabbing the medium and incubated at 30 °C for 7 days. After incubation, the tubes were removed from the incubator and placed in the refrigerator at 4°C for 30 minutes. After refrigeration, if the surface of the medium is liquid or fluid then it indicates gelatin hydrolysis.

Hydrogen sulfide, Motility and Indole test: The SIM (Sulfide-Indole-Motility) agar tubes were inoculated with the isolates by stabbing the medium and incubated at 30 °C for 24 to 48 h. after incubation, the SIM medium was examined for presence or absence of a black precipitate along the line of stab incubation. A black precipitate of FeS indicates the production of H2S.

The same medium was used to check motility of the isolates. Motility is present when the growth of the culture is not restricted to the stab line of the inoculation. Growth of the nonmotile bacteria is confined to the line of inoculation.

Indole production was checked by adding 5 drops of Kovac's reagent to the SIM cultures. Development of red color at the top of the tube indicates the production of Indole.

Citrate: Simmons citrate agar slants inoculated with the isolates and incubated at 30 °C for 24 to 48 h. after incubation, the slants were examined for the presence or absence of growth and for any change in color from green to blue.

Urea hydrolysis: Stuart's urea broth was inoculated with the isolates and incubated at 30 °C for 24 to 48 h. Urease production is indicated by a bright pink color throughout the culture.

Nitrate reduction: Nitrate broth was inoculated with respective isolates and incubated at 30 °C for 24 to 48 h. after growth, add 0.5 ml of nitrate test reagent A and 0.5 ml

of test reagent B were added to each of the culture tubes and mixed. A distinct red color indicates the positive test.

3.6 16S rDNA gene sequencing and phylogenetic analysis

The genomic DNA was isolated from the pure culture according to the procedure described by Kepley et al., (2001) [213]. The quality of the isolated genomic DNA was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed (Figure 3.2). The fragment of 16S rDNA was amplified by PCR from the isolated DNA. The PCR amplicon was purified and further processed for the sequencing. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with forward and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. For strain 1R, **8F** (AGAGTTTGATCCTGGCTCAG) and **1492R** (CGGTTACCTTGTTACGACTT) primer used. While for strains 11Y, GF and 3YS, **704F** (AGATTTTCCGACGGCAGGTT) and **907R** (CCGTCAATTCCTTTRAGTTT) primers were used for forward and reverse sequencing. The 16S rDNA sequence was compared against NCBI GenBank database using the BLAST alignment search tool. The sequences were aligned using multiple alignment software program Clustal-W. The distance matrix was generated using RDP database, and the phylogenetic tree was constructed using MEGA 5 [214, 215].

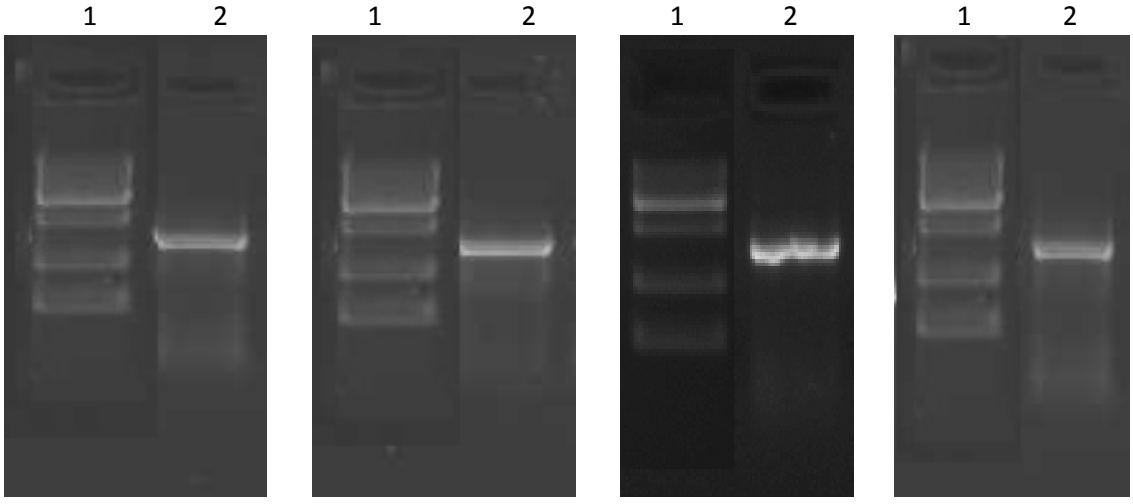


Figure 3.2: 1.2% Agarose gel showing single 1.5 kb and 16S rDNA amplicon. Lane 1: DNA marker (1 kb ladder); Lane 2: 16S rDNA amplicon (1500 bp).

3.7 Biodegradation Kinetic study:

Biodegradation kinetic study of the chlorophenols degradation was performed in batch mode. Chlorophenols are toxic and have inhibition effect on microorganism at higher concentration. Haldane/Andrew's substrate inhibition model was used to calculate the biokinetic parameters for degradation of chlorophenols [167].

$$R_s = \frac{R_m\, S}{K_s + S}\frac{K_i}{K_i + S} = \frac{R_m}{\left(1 + \frac{K_s}{S}\right)\left(1 + \frac{S}{K_i}\right)} \quad \text{(Eq. 3.1)}$$

Where, *Rs* and *Rm* are the actual and maximum rate of CP degradation (mg CP/L/h); *S* is the initial CP concentration (mg/L); K_s is the saturation constant (mg/L); K_i is the 2,4-DCP inhibition constant (mg/L).

For lower substrate concentration, the inhibition constant can be neglected. Hence, the above equation becomes,

$$R_s = \frac{R_m\, S}{K_s + S} \quad \text{(Eq. 3.2)}$$

In the linear form,

$$\frac{1}{R_s} = \frac{1}{R_m} + \frac{K_s}{R_m}\frac{1}{S} \quad \text{(Eq. 3.3)}$$

As the chlorophenol is toxic compound, there is a critical substrate concentration above which the removal rate decreases [181, 216]. Critical substrate concentration can be obtained by taking derivation of equation 3.1 with respect to *S*.

$$\frac{dR_s}{dS} = 0 \quad \text{(Eq. 3.4)}$$

Solving for S_{max} or S^*,

$$S_{max}\ or\ S^* = \sqrt{K_s K_i} \quad \text{(Eq. 3.5)}$$

S_{max} or S^*= critical substrate concentration after which removal rate decreases. The biokinetic constants were determined by the MATLAB 6.5 using the sum square error estimation. GraphPad Prsim-5 (trail version) and Origin software were also used for the estimation of biokinetic parameters.

3.8 Growth Kinetic study

Growth kinetic study was performed in batch mode. Haldane growth inhibition model was used to calculate the growth kinetic parameters [217].

$$\mu = \frac{\mu_m\, S}{S + K_s + \frac{S^2}{K_i}} \quad \text{(Eq. 3.6)}$$

Where, μ is the specific growth rate (hr^{-1}), μ_m is the maximum specific growth rate (hr^{-1}), k_s is the half saturation constant (mg/L), k_i is the substrate inhibition constant (mg/L), *S* is the substrate concentration (mg/L). The biokinetic constants were determined by the MATLAB 6.5 using the sum square error estimation. GraphPad Prsim-5 (trail version) and Origin software were also used for the estimation of biokinetic parameters.

3.9 Biodegradation study

Biodegradation experiments for all the chlorophenols compounds studied was performed in batch mode in 250 mL Erlenmeyer flask containing 50 mL MSM. The composition of the MSM is given in appendix 1. For higher chlorophenols such as 2,4-DCP, 2,4,6-TCP and PCP, 0.2 g/L of peptone is added to boost the initial growth of the microorganism. The medium was autoclaved at 121°C for 15 min. The chlorophenol compounds was added to the medium after autoclave by filter sterilizing using 0.22 µm syringe filter. The media was inoculated and incubated in rotary shaker at 30 °C and 120 rpm. The samples were taken at regular interval for the estimation of biomass, residual chlorophenols, chloride ions and other intermediates compounds.

3.10 Preparation of mixed consortium

In the present study, two different mixed microbial consortiums, one defined and another undefined, were used for the biodegradation study. The defined mixed microbial consortium was prepared by mixing the four different isolated pure strains. The four different strains used were *B. endophyticus* strain CP1R (KM259919), *Bacillus cereus* strain 3YS (KM522855), *Kocuria rhizophila* strain 11Y (KM522854), *Pseudomonas aeruginosa* strain GF (KM259920). Genbank accession numbers for four strains were shown in the corresponding brackets. All the four strains were equally mixed and used for the biodegradation experiments.

The second microbial consoritum used in the study was undefined and directly used for the biodegradation study after acclimatization. The mixed microbial consortium used in the study was isolated from the sludge and soil samples collected from both the sources as mentioned above. For enrichment and acclimation, 10 gm of sludge and soil collceted from both sources, were mixed in equal proportion and added to 100 mL of MSM containing peptone (1 to 0.1 g/L) and 2,4-DCP (20 to 250 mg/L) and incubated at 30 °C and 120 rpm in rotary shaker for a period of 6 month.The enriched culture was transferred to fresh MSM at every 15 days with increasing concentration of 2,4-DCP. The mixed consortium resulted after acclimatization period is used in the present study for biodegradation.

Both mixed microbial consoritum were acclimatized to 2,4,6-TCP (10 to 50 mg/L) and PCP (10 to 25 mg/L) for another two months in MSM medium. This resulting consoritums were used for 2,4,6-TCP and PCP biodegradation studies.

3.11 Bioreactor study

The isolated microorganisms were tested for their ability to degrade the chlorophenols in continuous bioreactor system. Bioreactor study is important to understand the practical application of the microorganism for bioremediation purpose. There are different parameters that have impact on biodegradation efficiency which has to be evaluated before application of microorganisms for bioremediation. Such factors include the substrate concentration, loading rate, hydraulic retention time, presence of biogenic substrate, pH, temperature, shock load, presence of other chlorophenol compounds and nutrient. In the present study, three different

bioreactors including two packed bed biofilm reactors and an airlift inner loop bioreactor were built in laboratory to study the effect of different parameters on biodegradation of chlorophenols.

3.11.1 Bioreactor medium

The bioreactors were fed with the mineral salt medium (MSM) containing corresponding chlorophenols and peptone as carbon and energy source. The composition of the MSM (modified DSMZ-465) as was mentioned in Appendix 1. The pH of the medium was adjusted to 7.35 using 1M HCl and 1M NaOH. For bioreactor study, the medium was prepared using the filtered tap water.

3.11.2 Packed bed biofilm reactor- 1 (PBBR-1)

A packed bed biofilm reactor- 1 (PBBR-1) was made from the Perspex glass (Figure 3.3, 3.5a, 3.6a, 3.6b). The diameter of the bioreactor was 5 cm and height was 60 cm. The conical shape below the reactor has top diameter of 5 cm and height of 6 cm. The ceramic disc (90 – 150 µm pore size) of 5 cm diameter was used as air diffuser. Ceramic balls ($\varnothing$ = 6 mm) was used for biofilm adhesion and was filled up to 25 cm height in the bioreactor. Ceramic balls are thermally stable, easily available, cheap and inert in nature that makes them quality material for biofilm formation. The chemical and physical properties of the ceramic balls used have shown in table 3.1 and 3.2, respectively. The ceramic balls were washed with diluted sulphuric acid and afterwards washed thoroughly with water. After drying in oven at 100 °C, the ceramic balls were autoclaved at 121 °C for 15 min before adding them to bioreactor. The total volume of the bioreactor was 600 mL in which the ceramic balled occupied the 393 mL and the conical shape has occupied 50 mL volume. The working volume of the bioreactor was 450 mL. The medium was fed to bioreactor using gravitational flow and the flow rate was controlled by peristaltic pump. Air flow rate used during the study was 0.1 LPM. The bioreactor was stored at room temperature (32 ± 3 ºC).

Table 3.1: Chemical composition of ceramic beads

Compound	Typical value
SiO_2	68.9%
Al_2O_3	24.1%
K_2O	2.8%
Na_2O	1.3%
CaO	0.6%
MgO	0.8%
Fe_2O_3	0.5%
TiO_2	0.7%
Leachable Iron	Nil

Table 3.2: Physical properties of ceramic beads

Physical properties	Typical value
Water Absorption (By weight)	0.33%
Apparent Porosity (By Volume)	<1%
Particle Density (Material Piece Density)	2.3 gm/cc
Hardness	7 Mohs scale
Working Temp	1000 °C
Maximum to Minimum diameter ratio	<1.25 mm

3.11.3 Packed bed biofilm reactor- 2 (PPBR-2)

The second packed bed biofilm reactor- 2 (PBBR-2) was made from the Perspex glass (Figure 3.4, 3.5b, 3.6a). The diameter and height of the bioreactor were5 cm and 100 cm respectively. The ceramic disc (90 – 150 µm pore size) of 5 cm diameter has used as air diffuser which is located 2 cm above from the bottom of the reactor. Ceramic balls ($Ø$ = 6 mm) were used for biofilm adhesion and filled up to 35 cm height in the bioreactor. The working volume of the bioreactor was 750 mL. The medium was fed to bioreactor using gravitational flow and the flow rate was controlled by peristaltic pump. Air flow rate used during the study was 0.2 LPM. The bioreactor was stored at room temperature (32 ± 3 ºC).

The PBBR-2 was used for biodegradation of 3-CP and 4-CP by the defined mixed microbial consortium.

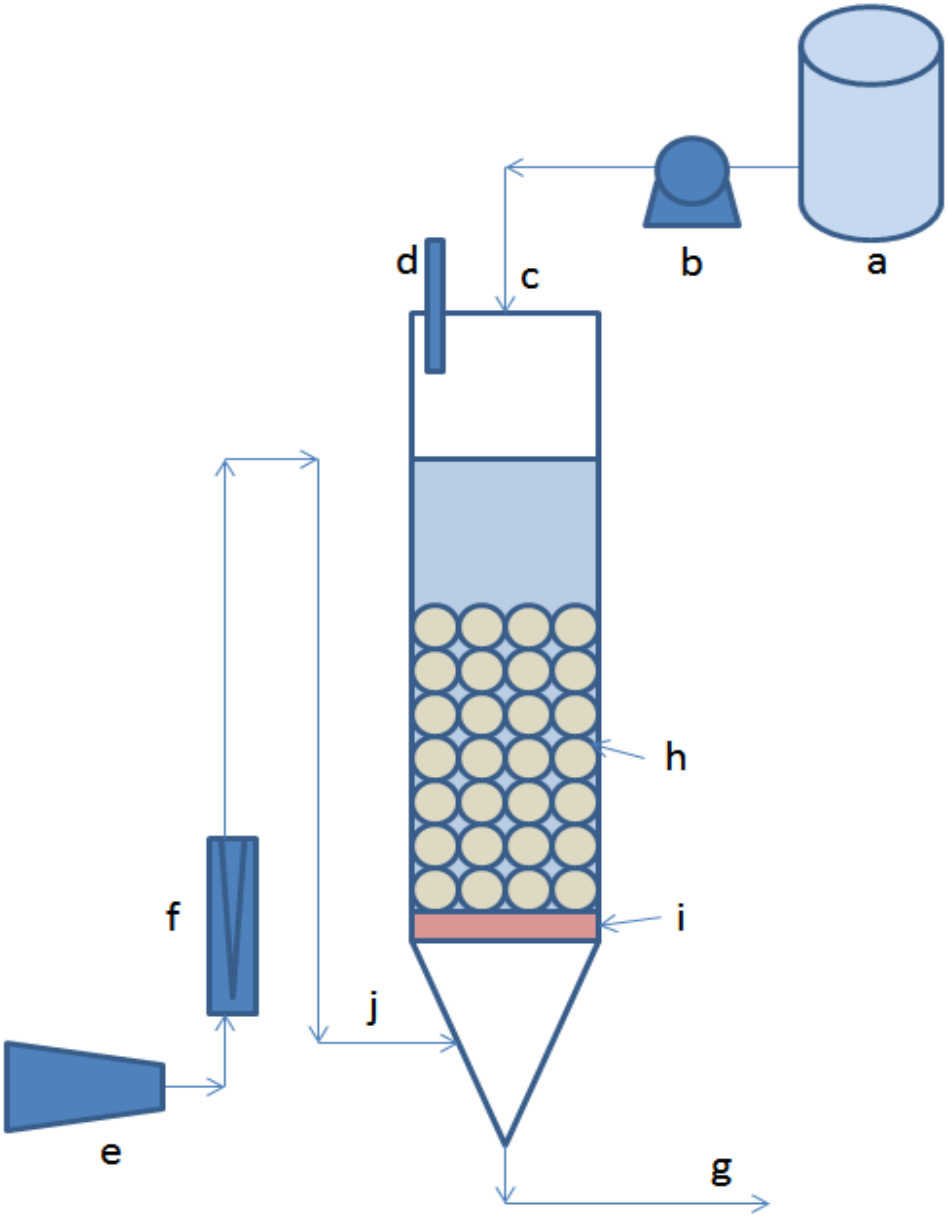


Figure 3.3: A schematic view of packed bed biofilm reactor- 1 (PBBR-1). (a) Feed tank, (b) Peristaltic pump, (c) Influent, (d) Air outlet and pH probe, (e) Air compressor, (f) Air rotameter, (g) Drainage or Effluent, (h) Packed bed, (i) Air diffuser, (j) Air inlet.

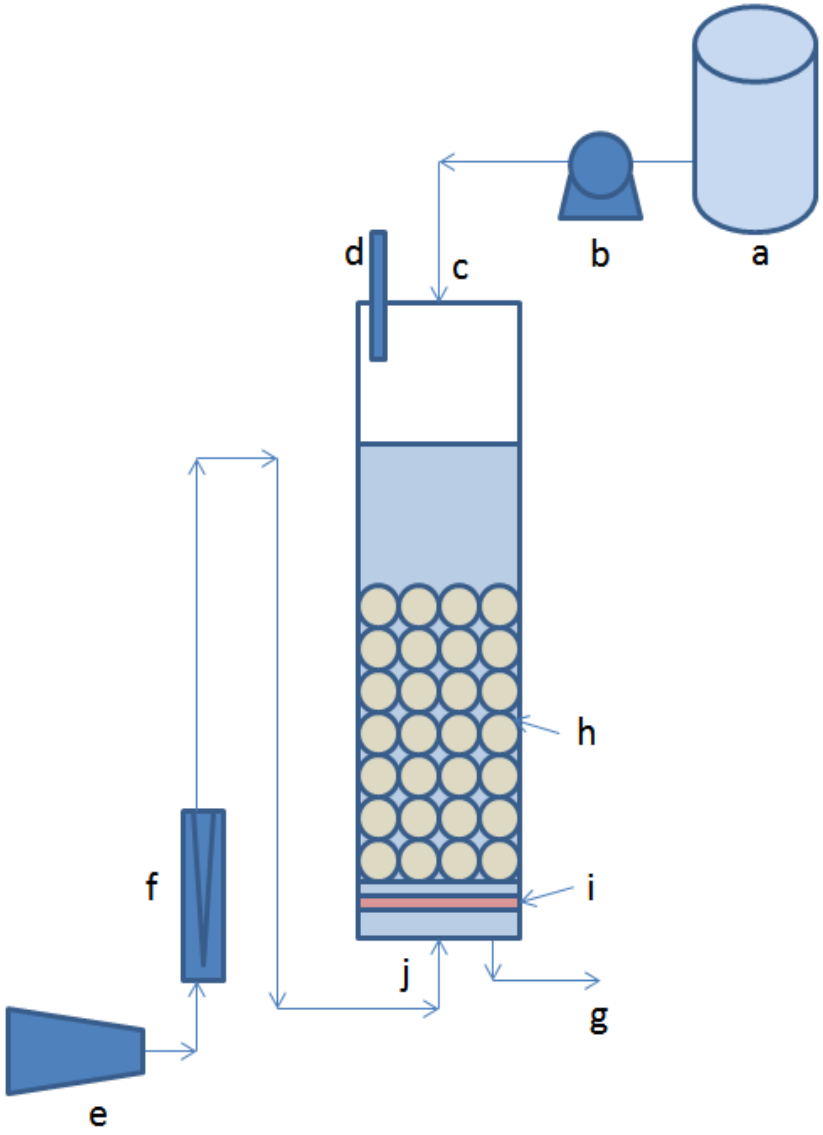


Figure 3.4: A schematic view of packed bed biofilm reactor- 2 (PBBR-2). (a) Feed tank, (b) Peristaltic pump, (c) Influent, (d) Air outlet and pH probe, (e) Air compressor, (f) Air rotameter, (g) Drainage or Effluent, (h) Packed bed, (i) Air diffuser, (j) Air inlet.

Figure 3.5: Packed bed biofilm reactor (a) PBBR-1 (b) PBBR-2

Figure 3.6: (a) Experimental setup for both Packed bed biofilm reactor (Left: PBBR-1; Right: PBBR-2) (b) Packed bed biofilm reactor- 1 (PBBR-1) during biofilm formation

3.11.4 Airlift inner loop reactor (ALR)

An airlift inner loop reactor made of Perplex glass was used throughout the experiment. The outer core has a dimension of 18 * 60 cm with a concentrically located inner draft tube of 9.5 * 35 cm. The working volume of the reactor is 12 L. The three metallic clamps supported the inner draft tube. The ceramic disc (90 – 150 µm pore size) with 9 cm diameter located below the inner tube was used as air sparger. A schematic view and the experimental setup of the reactor has shown in figure 3.7 and figure 3.8 respectively. The medium was fed to the bioreactor using gravitational flow and the flow rate was controlled by the peristaltic pump. Air flow rate used during the study was 4 LPM. The bioreactor was stored at room temperature (32 ± 3 ºC).

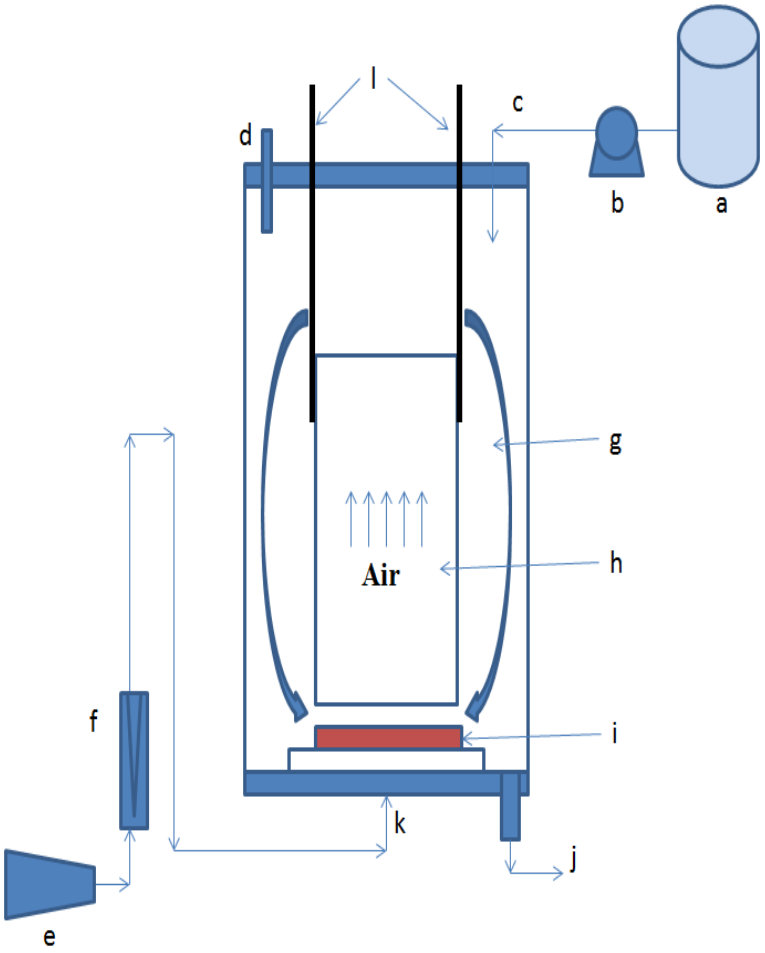


Figure 3.7. A schematic view of an airlift inner loop bioreactor (ALR). (a) Feed tank, (b) Peristaltic pump, (c) Influent, (d) Air outlet and pH probe, (e) Air compressor, (f) Air rotameter, (g) Downcomer, (h) Riser, (i) Air diffuser, (j) Drainage or Effluent, (k) Air input, (l) Metal rod supporting inner draft tube.

Figure 3.8: Airlift inner loop reactor (a) ALR during the start-up of the 4-chlorophenol biodegradation (b) ALR medium turned to yellow color due to accumulation of 5-chloro-2-hydroxymuconic semialdehyde during 4-chlorohenol biodegradation

Figure 3.9: Air compressor and air distributor used for air supply to all three bioreactors

CHAPTER – IV

RESULTS AND DISCUSSION

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RESULTS AND DISCUSSION

4.1 ISOLATION AND CHARACTERIZATION

The present section discusses enrichment, acclimatization and isolation of pure microorganisms in detail. It also presented the morphological, biochemical and molecular characterization of the isolated microorganisms.

4.1.1 Enrichment and acclimatization

The sludge and soil samples were collected from the two different sites, one from dye industry and another from steel plan, as mentioned in the previous chapter. The microorganism presents in the samples were enriched first and then acclimatized to the chlorophenols stepwise. Enrichment step is necessary to increase the growth of the present microorganism by providing carbon and nitrogen source. And acclimatization step is necessary, as microorganisms present in the samples are given time to adapt to new environment. Bacteria are very diverse in nature, easily adapted to new environment with time and change their metabolic expression according to new nutrient condition.

For enrichment, 10 gm of soil and sludge collected from both sites were added to 50mL nutrient broth (1x) containing 20 mg/L 2,4-DCP and incubated at 30 °C and 120 rpm for 7 days. After 7 days of incubation the culture was centrifuged at 10,000 rpm for 10 min and the cell pallet was resuspended in the series of diluted nutrient broth (0.5x, 0.1x, 0.01x) with 20 mg/L 2,4-DCP. After one month of enrichment, the culture was transferred to mineral salt medium containing 1 g/L peptone and 20 mg/L 2,4-DCP for acclimatization process. After every 15 days, the cultures were transferred to the fresh MSM medium. The peptone concentration was decreased from 1 g/L to 0.2 g/L and 2,4-DCP concentration was increased gradually from 20 mg/L to 200 mg/L over a period of six months.

4.1.2 Screening and isolation

During the acclimatization process, 0.1 mL of the sample from each sample was transferred to the MSM agar plates containing 0.2 g/L peptone and 50 mg/L 2,4-DCP. Isolation of pure bacterial strains from the final acclimated culture was done by using serial dilution technique and repeated streaking on MSM agar plates. There were 20 different bacterial culture isolated and tested for their ability to degrade 2,4-DCP (50 mg/L) individually in MSM medium. After final acclimation and 2,4-DCP degradation experiment, eight different bacteria were selected based on the morphological appearance, tolerance and degradation potentiality (Table 4.1). These eight strains were used for 2,4-DCP tolerance and degradation experiments at higher concentration, and from there three bacterial strains (1R, GF and 11Y) were selected for biodegradation experiment of chlorophenols.

Table 4.1: Growth of the isolated bacteria on MSM containing 2,4-DCP as sole carbon and energy source

Strain code	Growth of bacteria observed on MSM containing 2,4-DCP as sole carbon and						
	20 mg/L	50 mg/L	100 mg/L	150 mg/L	200 mg/L	250 mg/L	300 mg/L
1R	+	+	+	+	+	+	+
1S	+	+	+	-	-	-	-
3YS	+	+	+	+	+	+	-
5	+	+	+	+	+	-	-
11Y	+	+	+	+	+	+	+
GF	+	+	+	+	+	+	+
3WY	+	+	+	+	-	-	-
3WW	+	+	+	+	+	-	-

+ Growth; – No growth

4.1.3 Morphological and biochemical analysis

Morphological and biochemical analysis of the four selected isolates have been done according to bergey's manual (Table 4.2).

Table 4.2: Biochemical analysis of the isolated strains

	1R	**11Y**	**GF**	**3YS**
Shape	Rod	Round	Rod	Rod
Colony characteristic	Round, white colored	Round, dark yellow colored	Round with extracellular secretion, Cream/brown colored	Round with extracellular secretion, white colored
Gram test	+ve	+ve	+ve	+ve
Endospore	Round	No	No	Round
Motility	Non motile	Non motile	Non motile	Non motile
Gelatine	-ve	-ve	-ve	-ve
Starch	-ve	-ve	+ve	-ve
Casein	-ve	-ve	-ve	-ve
Urea	-ve	-ve	+ve	-ve
Indole	-ve	-ve	-ve	-ve
Sulpher	-ve	-ve	-ve	-ve
Citrate	-ve	-ve	-ve	-ve
MR	-ve	-ve	-ve	+ve
VP	-ve	-ve	-ve	+ve
Nitrate	+ve	-ve	+ve	+ve
Glucose	+ve	+ve	+ve	-ve

Fructose	+ve	+ve	+ve	-ve
Maltose	+ve	+ve	-ve	-ve
Mannose	+ve	+ve	-ve	-ve
Mannitol	+ve	+ve	-ve	-ve

4.1.4 16S rDNA gene sequencing and molecular analysis

Using PCR, 16S rDNA gene was amplified, sequenced and consensus sequences of 1330, 1430, 1493 and 1425 bp for strains 1R, 11Y, GF and 3YS respectively, were generated from forward and reverse sequence data using aligner software. All the sequences were submitted to GenBank and accession numbers generated. The sequences were aligned against the GenBank database using BLAST alignment tool. Based on maximum identity score, first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree (Figure 4.1, 4.2, 4.3 and 4.4) was constructed using MEGA 5 software based on 16S rDNA sequence using Neighbor-Joining method. Table 4.3 summarizes the strain identification and corresponding Genbank accession number obtained.

Table 4.3: 16S rDNA analysis and GenBank accession number of the isolates

Strain code	**Strain name**	**GenBank accession number**
1R	*Bacillus endophyticus* CP1R	KM259919.1
11Y	*Kocuria rhizophila* 11Y	KM522854.1
GF	*Pseudomonas aeruginosa* GF	KM259920.1
3YS	*Bacillus cereus* 3YS	KM522855.1

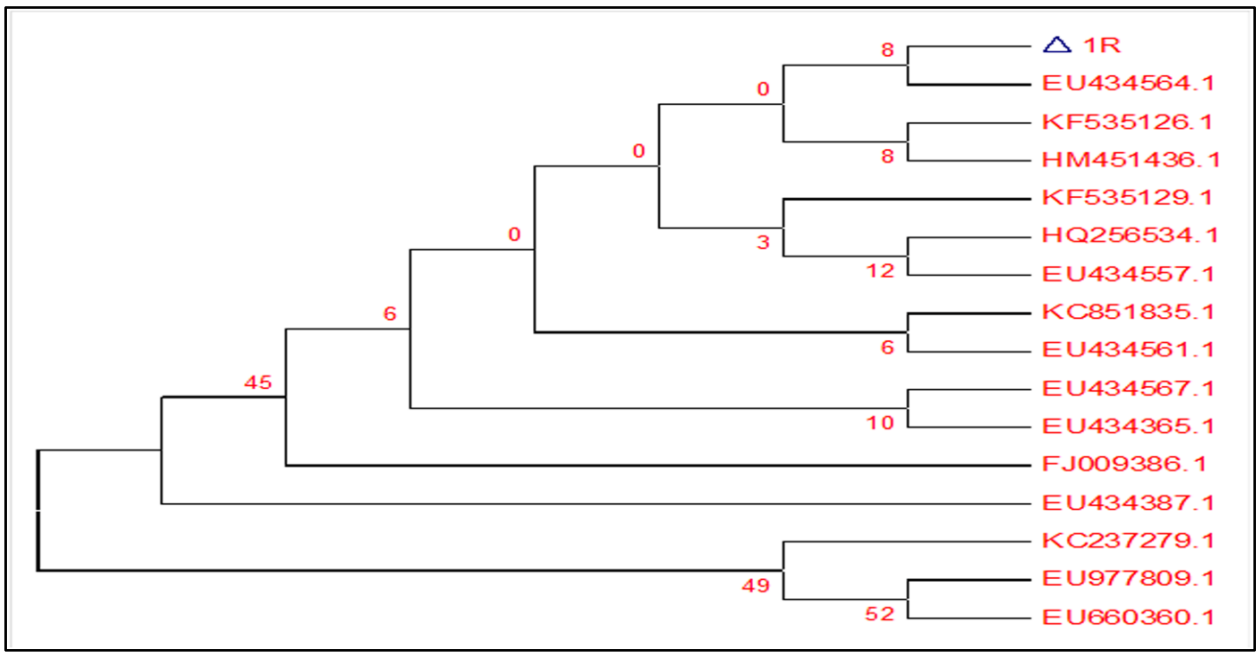


Figure 4.1: Phylogenetic tree showing evolutionary relationship of isolate '1R' with 15 taxa using Neighbor-Joining method.

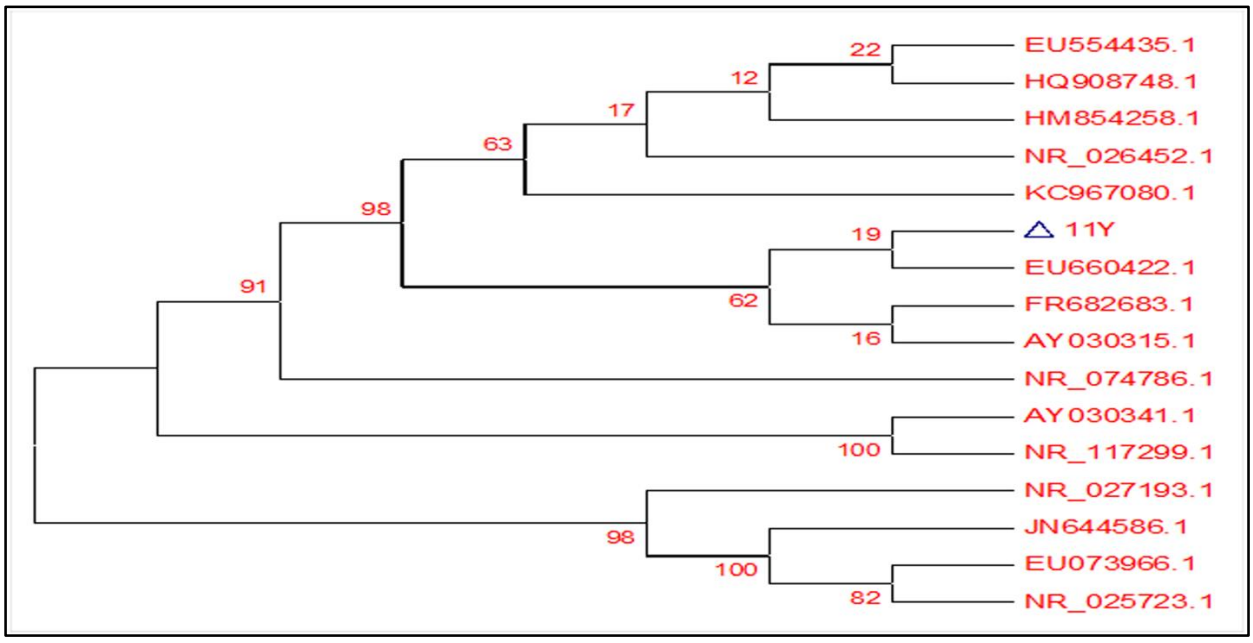


Figure 4.2: Phylogenetic tree showing evolutionary relationship of isolate '11Y' with 15 taxa using Neighbor-Joining method.

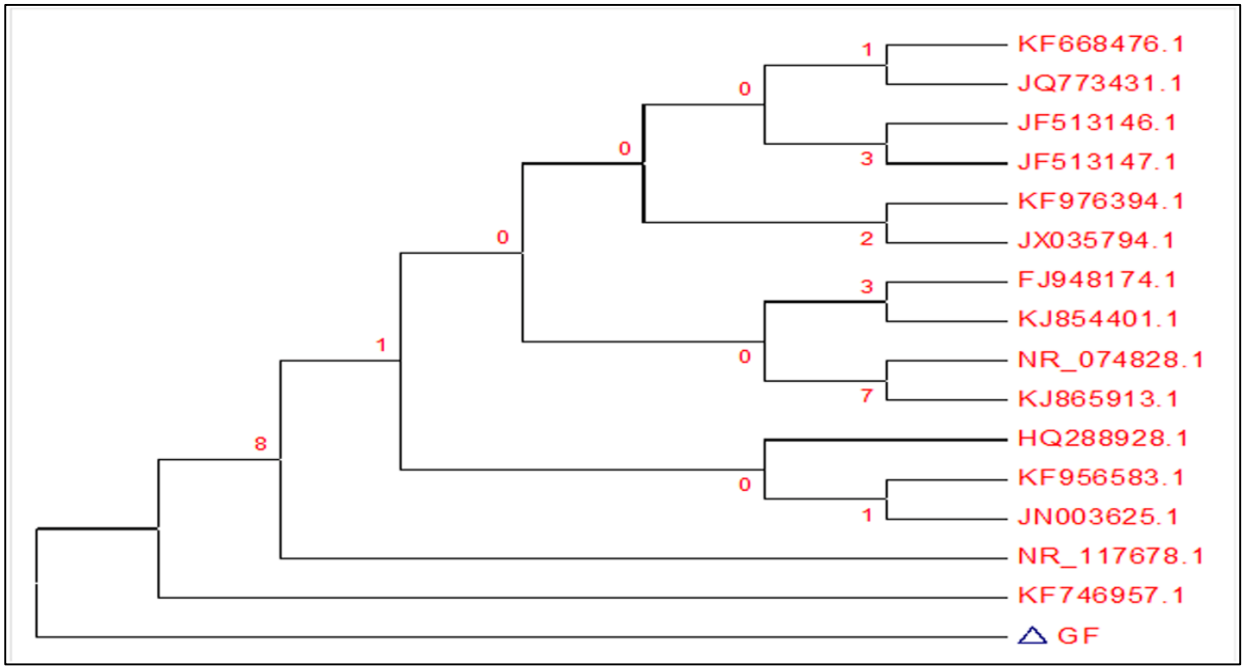


Figure 4.3: Phylogenetic tree showing evolutionary relationship of isolate 'GF' with 15 taxa using Neighbor-Joining method.

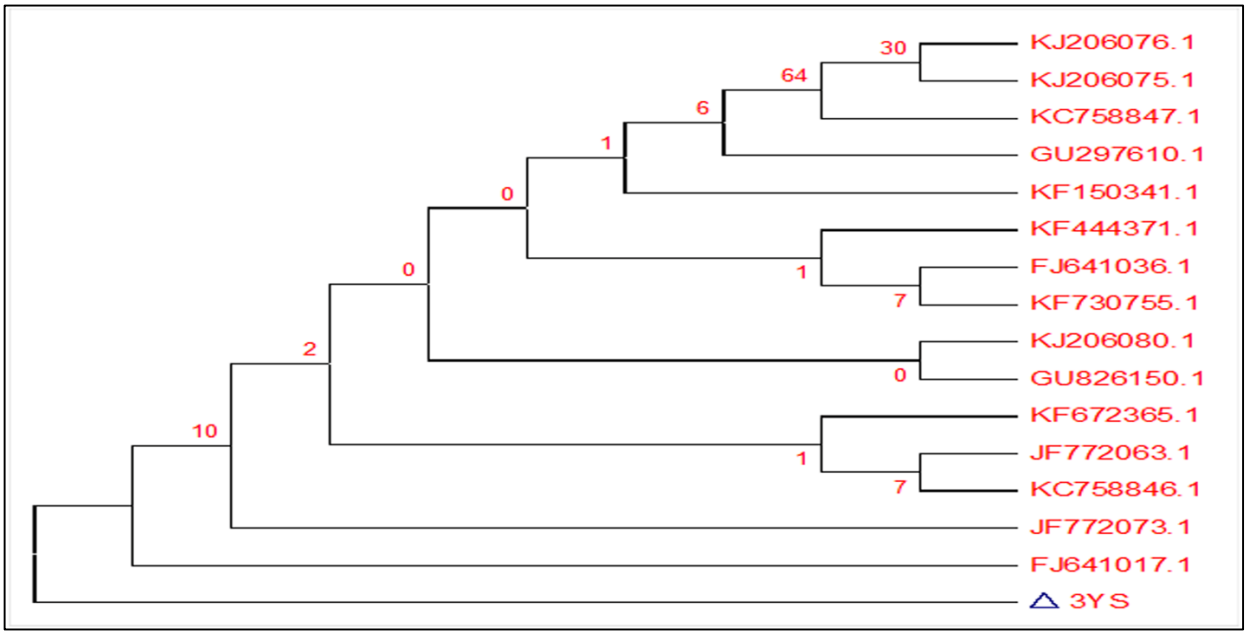


Figure 4.4: Phylogenetic tree showing evolutionary relationship of isolate '3YS' with 15 taxa using Neighbor-Joining method.

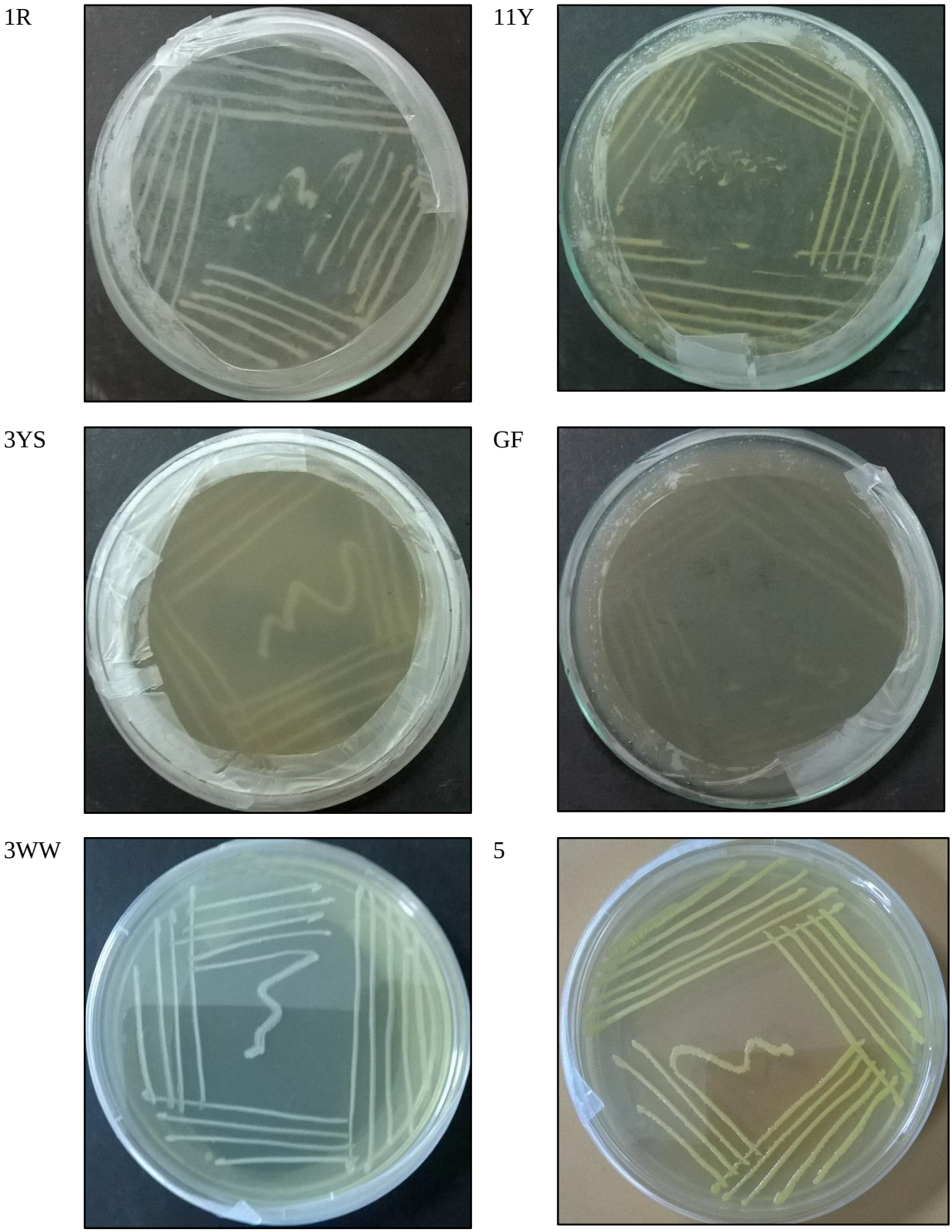


Figure 4.5: Isolated pure bacteria in mineral salt medium containing 50 mg/L of 2,4-DCP.

4.2 Biodegradation of chlorophenols by *Bacillus endophyticus* strain CP1R: Optimization, Kinetic and Cometabolism

In the present section, the biodegradation of six different chlorophenols including 2-CP, 3-CP, 4-CP, 2,4-DCP, 2,4,6-TCP and PCP by ***Bacillus endophyticus* strain CP1R** was studied. Also, the growth and biodegradation kinetics of the chlorophenol biodegradation were studied. The optimization of four different environmental parameters was performed using the response surface methodology to achieve the maximum biodegradation of 2,4-DCP. At last, the capability of the isolate to degrade the 2,4-DCP in the presence of all the three monochlorophenols were evaluated.

The isolate didn't show any degradation of 2,4,6-TCP and PCP as sole carbon and energy while it has shown only negligible degradation for 3-CP and 4-CP. However, the strain was able to removal 2-CP and 2,4-DCP efficiently. The effect of glucose as secondary carbon source on removal efficiency of 2,4-DCP was evaluated.

4.2.1 Biodegradation and kinetic of 2,4-DCP by strain CP1R

The biodegradation of 2,4-DCP at a different initial concentration of 25, 50, 100, 200 mg/L by the *Bacillus endophyticus* strain CP1R was carried out for 264 h as sole carbon and energy source. Figure 4.6 and 4.7 shows the residual concentration of 2,4-DCP and growth of the *Bacillus endophyticus* strain CP1R respectively. The abiotic loss of 2,4-DCP due to evaporation was negligible in control flask. The isolate was able to degrade 2,4-DCP completely up to 50 mg/L as shown in figure 4.6. It has completely degraded 25 and 50 mg/L 2,4-DCP within 120 and 216 h respectively, while it has shown 75 and 59% degradation of 100 and 200 mg/L 2,4-DCP as sole carbon and energy source. From figure 4.7, It can be observed that the growth of *B. endophyticus* strain increases with substrate concentration up to 50 mg/L and afterwards it starts to decreases due to inhibition effect imposed by high concentration of 2,4-DCP. The biomass growth decreases and the lag period increases with increase in 2,4-DCP concentration due to the toxic effect.

Figure 4.8 shows the effect of initial 2,4-DCP concentration on the removal rate. The removal rate increases with increase in initial 2,4-DCP concentration up to 200 mg/L. The overall maximum removal rate obtained was 0.396 mg DCP/h/L (Equation 3.2). The pH of the medium was dropped to 6.6 to 6.85 from an initial value of pH 7.0±1 at the end of the experiment, because of the formation of hydrochloric acid due to the release of chloride ion.

The release of chloride ion was observed during the biodegradation of 2,4-DCP. However, the exact amount of chloride ion released was not analyzed. Electrospray ionization mass spectroscopy analyzed the presence of metabolites during the biodegradation of 2,4-DCP. The sample was acidified with 1M HCl and extracted with ethyl acetate. The extracted sample was analyzed for metabolites (Figure 4.9). Different m/z peaks were observed in the final sample that represents the biodegradation products. The highest peak m/z=370 was related to the conjugation product of two sodium 2,4-dichlorophenoxy ion. The multiplet (296, 298.9, 301 m/z) correspond to above conjugation with the release of two chloride ion and (280 m/z) with the release of –OH ion. The m/z peaks at 201, 199, 198, 180, 181, 141,

and 79 corresponding to 3,5-dichlorocatechol were identified [218]. Other m/z peaks (254, 256, 258, 242, 202, 180, 127, 112, and 101) related to the degradation product or conjugate fragment was also identified. The multiplets (170, 152, 129, 111, and 101) identified as monochlorophenol and its conjugate fragments.

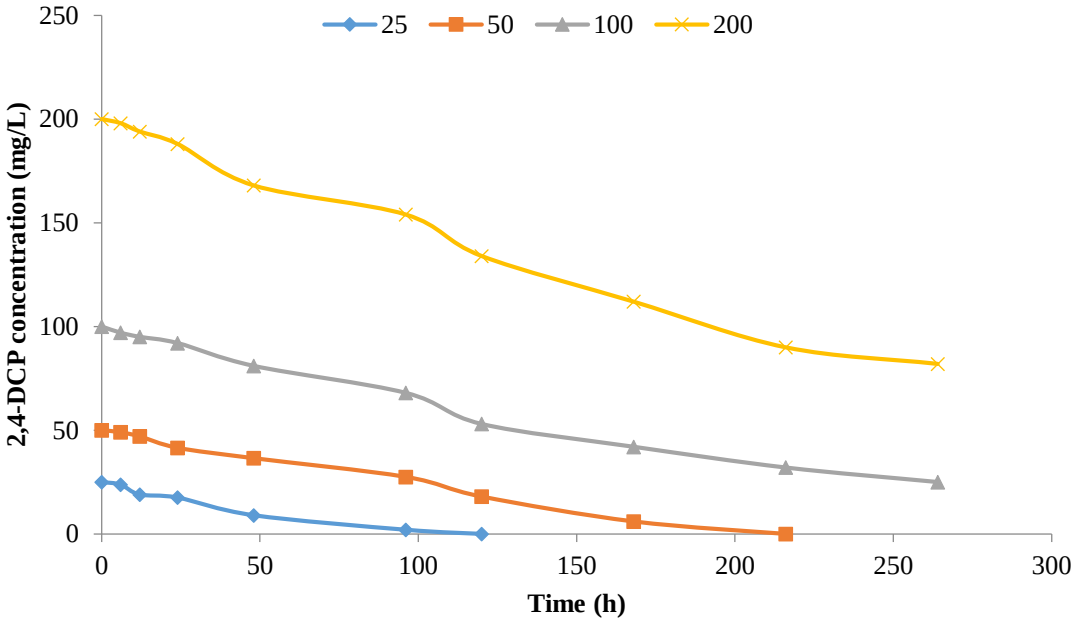


Figure 4.6: The biodegradation profile for 2,4-dicholrophenol by *B. endophyticus* CP1R at different initial substrate concentration.

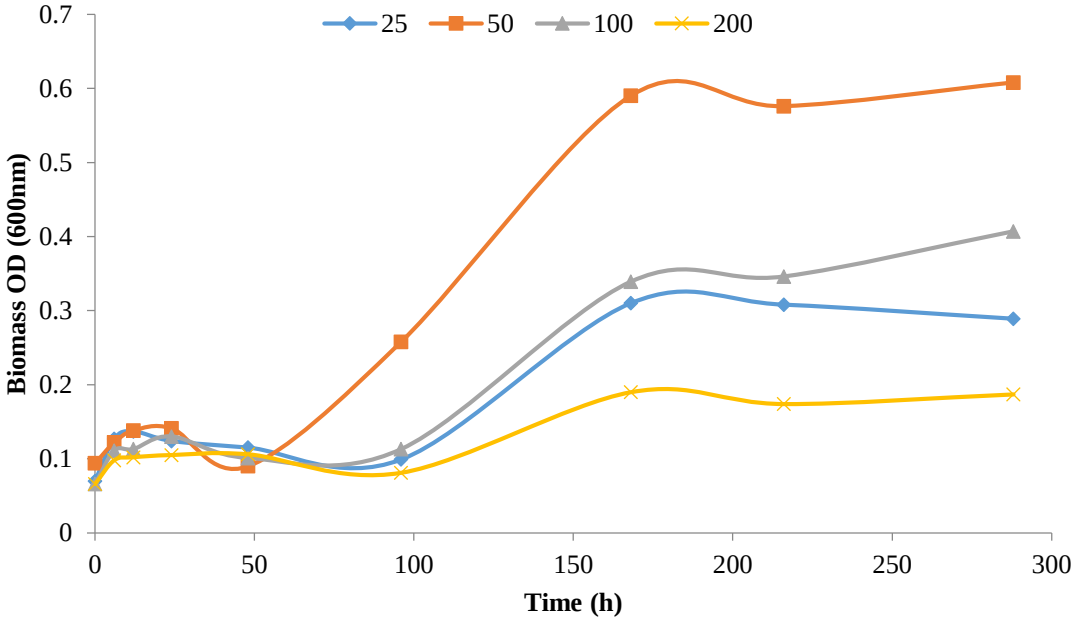


Figure 4.7: Biomass growth profile of *B. endophyticus* CP1R at different initial 2,4-DCP concentration.

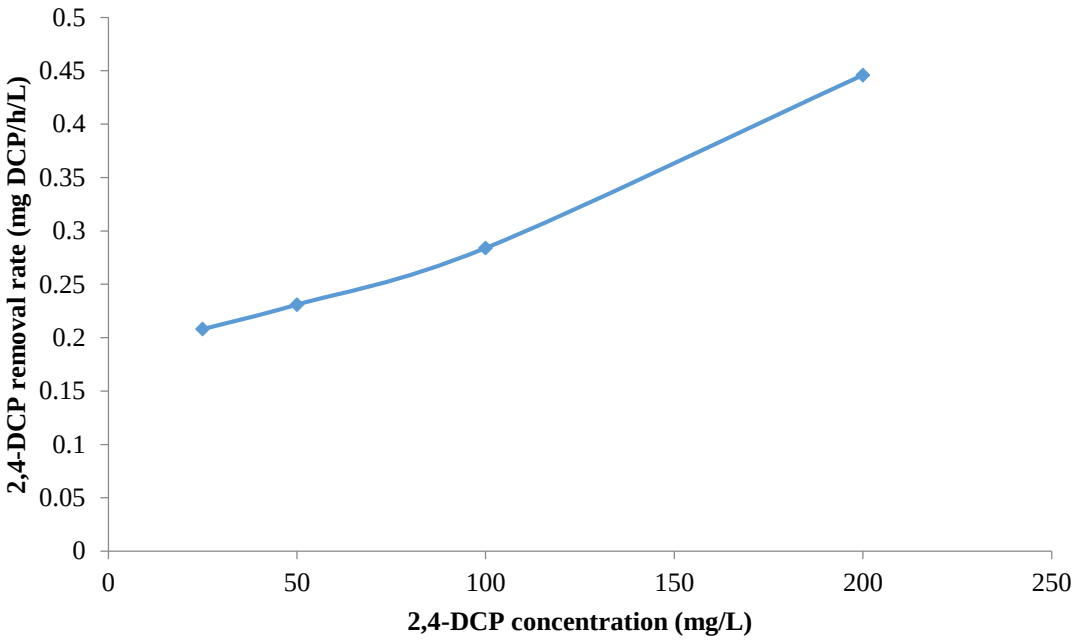


Figure 4.8: The effect of initial 2,4-DCP concentration on the removal rate by *B. endophyticus* CP1R.

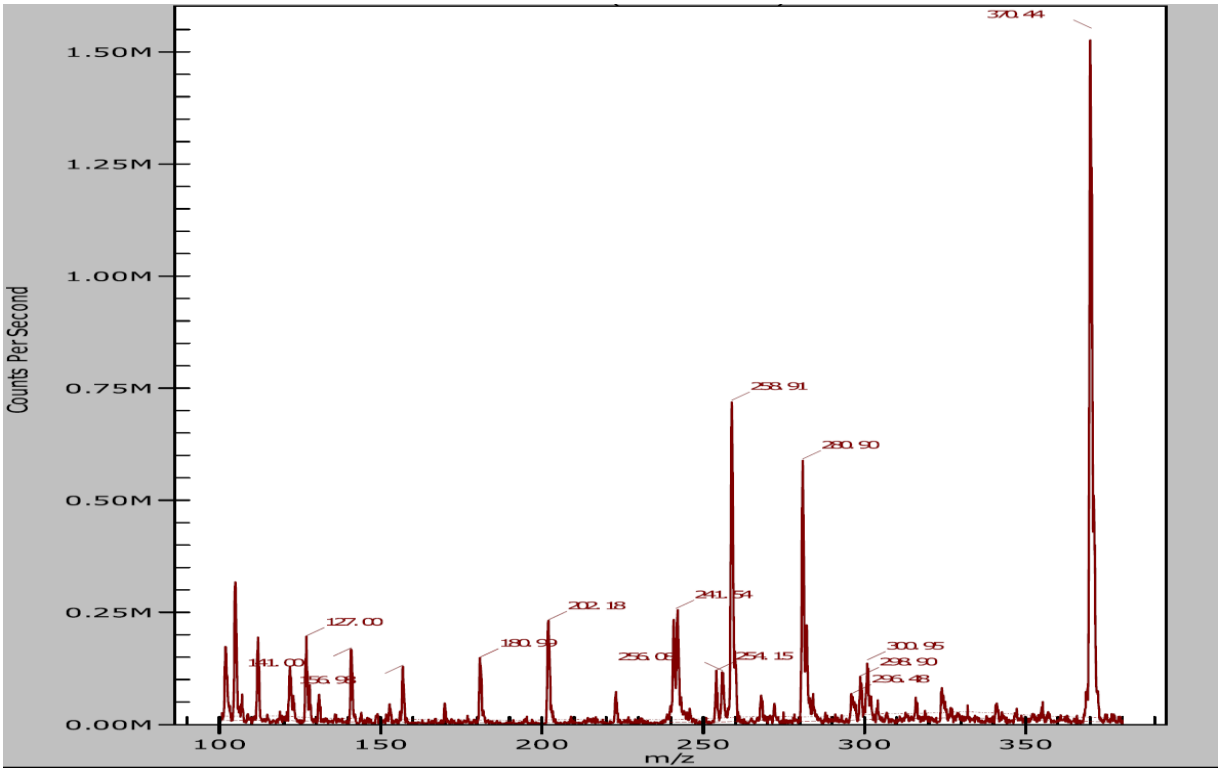


Figure 4.9: Mass spectrum of 2,4-DCP (50 mg/L) biodegradation products by *B. endophyticus* CP1R.

4.2.1.1 Effect of glucose on 2,4-DCP degradation

2,4-DCP is toxic and inhibits the growth of the microorganisms. This inhibition effect can be diminished to some extent by the addition of secondary carbon sources such as glucose, peptone, yeast extract, etc. which are primarily utilized by a microorganism [10]. Figure 4.10 shows the effect of glucose concentration (0.5, 1, 1.5 and 2 g/L) on the degradation of 50 mg/L of 2,4-DCP by *Bacillus endophyticus* CP1R. The efficiency of 2,4-DCP degradation was greatly increased in the presence of glucose, and more than 75% degradation of 50 mg/L of 2,4-DCP was observed within 96 h. The maximum of 98% degradation of 2,4-DCP was observed with 1.5 g/L of glucose within 96 h. There was no further increase in degradation rate observed with 2 g/L of glucose. While, without glucose, the isolate has shown only 45% degradation of 50 mg/L of 2,4-DCP within 96 h. The addition of secondary carbon source such as glucose act as growth substrate and contributes to higher biomass growth which leads to the higher degradation rate of 2,4-DCP. Similar results of cometabolism of chlorophenols in the presence of additional carbon and nitrogen source were reported in several studies [207, 219, 220].

4.2.1.2 Growth kinetic study

Figure 4.11 shows the relation between specific growth rate and substrate concentration. From the figure, it has been seen that biodegradation of 2,4-DCP follows the substrate inhibition kinetics as specific growth rate decreases with increase in substrate concentration. The experimental data was fitted to Haldane substrate inhibition model (Equation 3.6) by non-linear least square regression method using Graph Pad Prism (Trial version) and Matlab 6.5. The biokinetic parameters value obtained were: maximum specific growth rate ($\mu_{m)}$ = 0.023 h^{-1}, half saturation constant (k_s) = 0.01 mg/L and substrate inhibition constant (k_i) = 47.5 mg/L. Therefore, the growth equation 3.6 for 2,4-DCP utilization by *Bacillus endophyticus* strain CP1R as sole carbon and energy source can be expressed as:

$$\mu = \frac{0.023\, s}{s + 0.01 + \frac{s^2}{47.5}}$$

The degradation of 2,4-DCP by other *Bacillus* sp. was also reported in several studies [11, 221]. The *Bacillus insolitus* strain has showed 42 and 51.7% degradation of 10 and 25 mg/L of 2,4-DCP after 16th day respectively. This strain was able to degrade less than 50% of 2,4-DCP up to 200 mg/L [221]. Herrera et al. (2008) reported 70 and 85% degradation of 3 mM 2,4-DCP by *Bacillus* consortium isolated from the polluted soil in the presence of NH_4Cl and KNO_2 nitrogen source respectively in 21 days [11]. Kargi and Eker (2005) reported the degradation of 50 to 750 mg/L of 2,4-DCP by *Pseudomonas putida* CP1. The percentage 2,4-DCP removal was declined from 35% at the lowest initial 2,4-DCP concentration of 51 mg/L to 13% at the highest initial 2,4-DCP concentration of 758 mg/L. The maximum rate of 2,4-DCP degradation was found 0.8 mg DCP/L/h at an initial 577 mg DCP/L and declined for higher initial DCP concentration [184]. In the present study, the isolate *Bacillus endophyticus* CP1R was able to completely degrade 2,4-DCP up to 50 mg/L and also it shows 75 and 59%

degradation of 100 and 200 mg/L of 2,4-DCP respectively. The degradation of 2,4-DCP obtained was high for pure culture and was in good agreement with the literature.

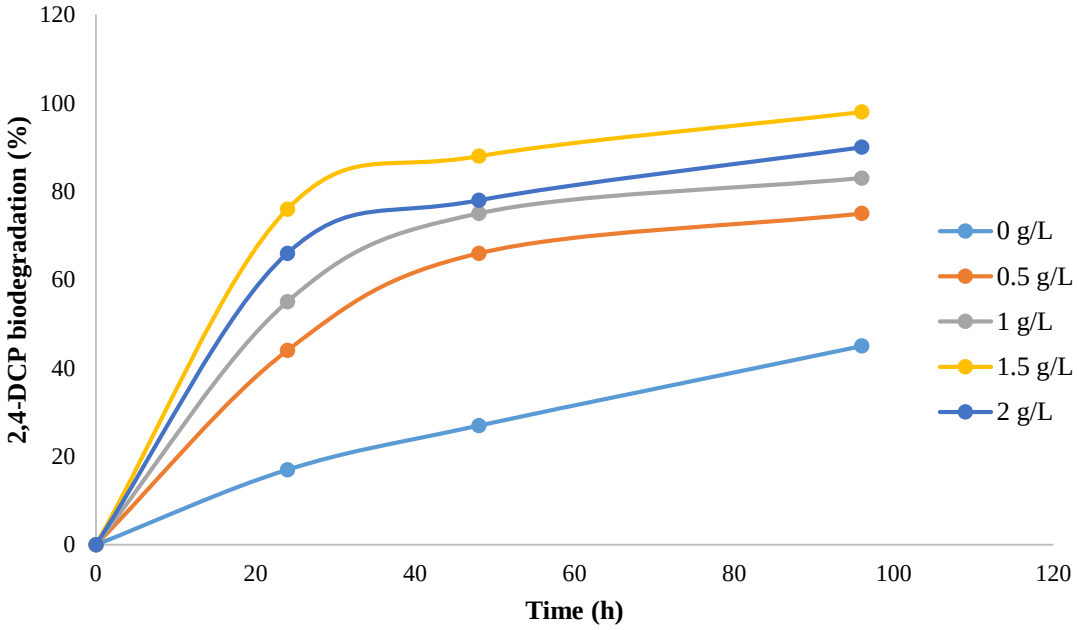


Figure 4.10: Effect of glucose concentration on 2,4-DCP (50 mg/L) degradation by *B. endophyticus* CP1R.

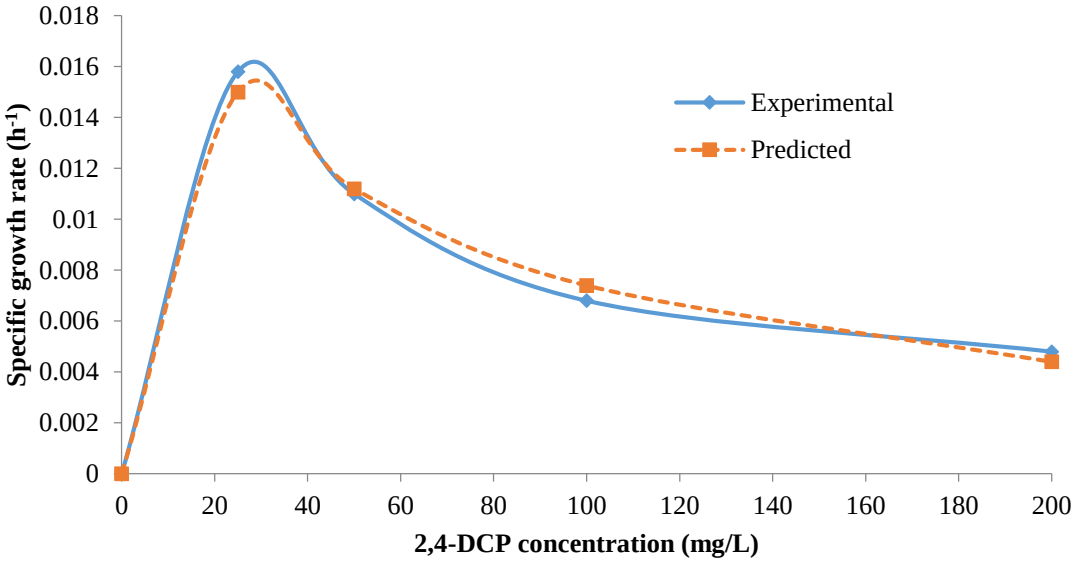


Figure 4.11: Effect of initial 2,4-DCP concentration on the specific growth rate of *B. endophyticus* CP1R.

Goswami et al. (2002) has reported the value of μ_m = 0.024 $hr^{-1}$and K_i = 81.34 mg/L for 2,4-DCP (10–50 mg/L) degradation by *Rhodococcus erythropolis* M1 (MTCC 3951) [179]. The biokinetic parameters obtained in this study were in good agreement with the literature. The low value of half saturation constant (K_s= 0.01) shows the high affinity of the isolate for 2,4-DCP, and the low inhibition constant indicates that the 2,4-DCP has high inhibition effect on the growth of the isolate. In this study, the maximum biodegradation rate observed was 0.394 mg DCP/L/h up to 200 mg/L of 2,4-DCP.

4.2.2 Optimization of experimental parameters to achieve maximum biodegradation of 2,4-DCP by B. endophyticus CP1R

In this study, optimization of various experimental parameters i.e. pH, temperature (ºC), inoculum size %(v/v) and ammonium sulphate concentration (g/L) at different levels were carried out to enhance the 2,4-DCP degradation by *Bacillus endophyticus* CP1R. The biodegradation kinetic parameters for 2,4-DCP were also calculated for the same at optimized condition. The central composite design of RSM was used to optimize the parameters. The independent variables and their corresponding levels used in the study have shown in Table 4.4.

Table 4.4: Independent variables and their corresponding levels used in the optimization study for *B. endophyticus* CP1R

Factors	Coded Unit	-α	-1	0	+1	+α
pH	X_1	5	6	7	8	9
Temperature (°C)	X_2	20	25	30	35	40
Inoculum Size[a] %(v/v)	X_3	2	4	6	8	10
$(NH_4)_2SO_4$ (g/L)	X_4	0	0.5	1	1.5	2

[a] One mL inoculum equals to ~11 mg dry biomass.

4.2.2.1 Optimization of experimental factors using RSM

The optimization of experimental factors for maximizing the 2,4-DCP biodegradation by *Bacillus endophyticus* CP1R was successfully performed as per central composite design. The effect of all four independent variables i.e. pH, temperature (°C), inoculum size %(v/v) and $(NH_4)_2SO_4$ (g/L) at different levels was analyzed on 2,4-DCP biodegradation. Table 4.5 summarized the experimental conditions and corresponding percent degradation obtained. The experimental data was analyzed in terms of the second order polynomial equation.

Table 4.5: Central composite design of experiment and % biodegradation of 2,4-DCP for *B. endophyticus* CP1R

Run Order	pH	Temperature (ºC)	Inoculum Size %(v/v)	$(NH_4)_2SO_4$ (g/L)	% Degradation	
					Experimental	Predicted
1	0	0	0	0	69	66.85
2	0	2	0	0	54	60.87
3	0	0	0	0	65	66.85
4	0	0	2	0	88	86.54
5	2	0	0	0	30	34.04
6	0	0	0	0	68	66.85
7	0	0	-2	0	46	50.04
8	1	-1	-1	1	22	19.95
9	0	0	0	0	68	66.85
10	0	-2	0	0	17	12.70
11	-1	-1	1	-1	52	50.66
12	-1	-1	1	1	42	45.95
13	1	1	1	-1	76	75
14	1	-1	1	-1	42	40.29
15	1	1	-1	-1	60	52.12
16	1	1	-1	1	54	56.66
17	-1	1	-1	1	48	45.79
18	1	-1	-1	-1	30	29.16
19	0	0	0	0	66	66.85
20	1	1	1	1	87	82.29
21	0	0	0	2	60	55.20
22	0	0	0	0	67	66.85
23	0	0	0	0	65	66.85
24	-1	1	1	-1	64	62.12
25	-1	1	1	1	69	71.16
26	-2	0	0	0	35	33.54
27	-1	1	-1	-1	43	39.5
28	1	-1	1	1	29	33.83
29	-1	-1	-1	1	30	32.33
30	0	0	0	-2	48	55.37
31	-1	-1	-1	-1	39	39.79

The value ofthe coefficient of determination (R^2) and adjusted R^2 for regression model is 0.963 and 0.932 which are close to 1 (Figure 4.12), explaining that the model sufficiently fits the data. The *f*-value and *p-value* were used to test the significance of the regression model. Higher *f*-value means that the model significantly explains the relation between the dependent and independent variables. The observed *f*-value for the regression model is 30.38 which is greater than critical *f*-value ($f_{0.05,14,16}$= 2.33) at a significant level of p=0.05 indicating that the regression model is significant and sufficiently explains all the variation. The critical $f_{0.05,4,16}$= 3 which is less than the calculated *f*-value implying that the linear and square terms of the regression model for 2,4-DCP degradation are significant. The $f_{0.05,6,16}$= 2.74 which is slightly lower than calculated f=6.38 implying the little less significance of the interaction terms of the regression model.

The analysis of variance (ANOVA) and the regression coefficient for 2,4-DCP degradation was summarized in Table 4.6 and 4.7 respectively. From the ANOVA table, it was conclusive that there was a significant interaction between the factors. The small *p*-values of interaction and square terms indicate that there is a curvature in the regression surface. Also, the small *p*-values for the linear and square terms indicate their significant contribution to the model.

From Table 4.7, it was concluded that the main effect of temperature and inoculum size was significant at an individual significant level of 0.05. The small *p*-value (p<0.05) for quadratic terms pH × pH, temperature × temperature and $(NH_4)_2SO_4$ × $(NH_4)_2SO_4$ concentration and also for the interaction terms $(NH_4)_2SO_4$ × $(NH_4)_2SO_4$, pH × temperature, temperature × inoculum size, temperature × $(NH_4)_2SO_4$ indicate their significance in the regression model. Thus, the regression model equation (in uncoded form) showing the effect of all four independent variable including interaction effect on 2,4-DCP biodegradation can be presented as below:

$$Y = -379.97 + 9.17X_2 - 6.23X_3 - 8.26X_1^2 - 0.3X_2^2 - 11.56X_4^2 + 1.16X_1X_2 + 0.29X_2X_3 + 1.37X_2X_4$$

Where, Y = 2,4-DCP biodegradation, X_1 is pH, X_2 is temperature (°C), X_3 is inoculum size %(v/v) and X_4 is $(NH_4)_2SO_4$ (g/L).

The interaction effect of two independent variables, while keeping other variables at middle point setting, on response was well illustrated by using the contour plots. The interaction effect of inoculum size and temperature was illustrated in figure 4.13(a). From the contour plot, it was seen that the percent degradation increases with increase in inoculum size and temperature. The response increases linearly with inoculum size whereas it was at maximum for temperature in the range 35-40 °C. The contour plot of temperature and pH was elliptical showing interaction effect on the response as shown in figure 4.13(b). The percent degradation was at maximum for the temperature around 35 °C and pH around 7.2 to 7.5.

The interaction effect of temperature and ammonium sulphate concentration was shown in figure 4.13(c). The contour plot is also elliptical indicating the significant

interaction between the variables. From the shape of the contour plot, it could be seen that the effect of ammonium sulphate concentration is less significant on response compare to temperature. By using the middle point setting, the optimum setting for maximum degradation obtained was 35 °C for temperature and around 1.4 g/L for ammonium sulphate concentration. The contour plot for the interaction between the other variables was circle indicating the less significant effect on response. The model adequacy was also checked by analysing the internally studentized residuals data which shows that all the residuals, except three, have values under 2. The normality plot of residuals shows that all the residuals fall along the straight line (Figure 4.14).

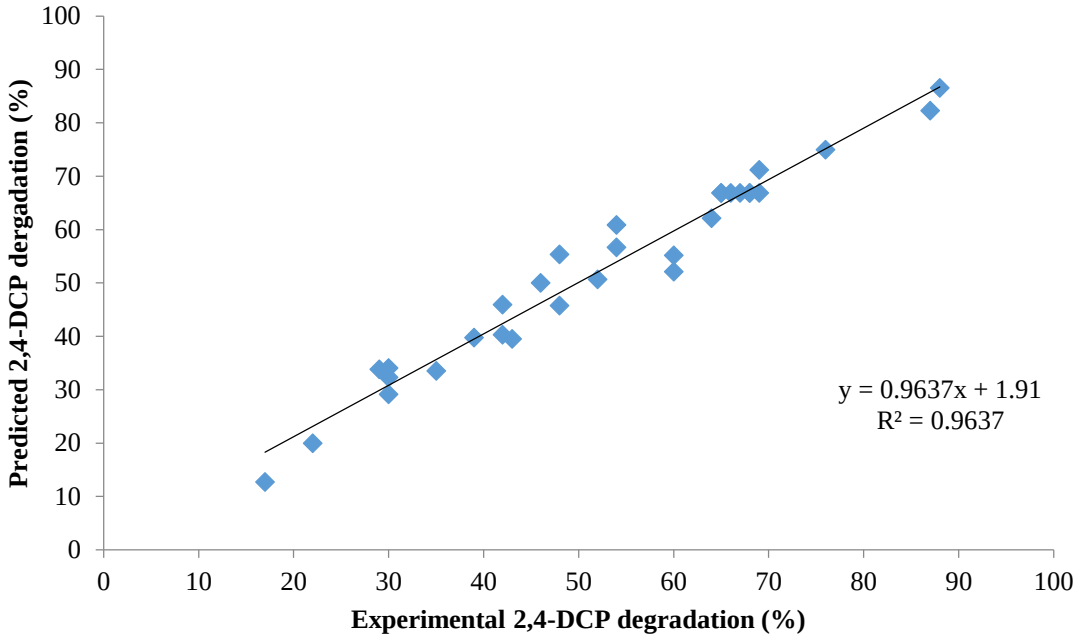


Figure 4.12: The Linear plot for actual versus predicted biodegradation of 2,4-dichlorophenol.

Using the desirability function, the optimum values of experimental parameters obtained were: pH 7.36, temperature 35.1°C, inoculum size 10 %(v/v) and $(NH_4)_2SO_4$ concentration 1.4 g/L. These optimum values were verified experimentally in batch mode using shake flask culture. The maximum of 100% degradation for 50 mg/L of 2,4-DCP within 384 h was observed which is up to 27% higher than un-optimized condition. Also, the strain was able the degrade high concentration of 2,4-DCP up to 400 mg/L at optimized condition without much inhibition effect that used to prevail after 200 mg/L of 2,4-DCP at the un-optimized condition.

Table 4.6: Analysis of Variance for % biodegradation of 2,4-DCP by *B. endophyticus* CP1R

Source	**DF**	**Seq SS**	**Adj MS**	**F**	**P**
Regression	14	9757.7	696.98	30.38	0
Linear	4	5478.8	1369.71	59.7	0
Square	4	3400.5	850.11	37.05	0
Interaction	6	878.4	146.4	6.38	0.001
Residual Error	16	367.1	22.94		
Lack-of-Fit	10	352.2	35.22	14.23	0.002
Pure Error	6	14.9	2.48		
Total	30	10124.8			
R-Sq = 0.963; R-Sq (adj) = 0.932					

DF- degree of freedom; Seq SS- sequential sum of squares; Adj MS- adjusted means square

Table 4.7: Regression coefficient for 2,4-DCP biodegradation by *B. endophyticus* CP1R

Term	**Coefficient**	**SE Coefficient**	**T**	**P**
Constant	-379.976	1.8105	36.928	0
X_1	81.6667	0.9778	0.128	0.9
X_2	9.17262	0.9778	12.316	0
X_3	-6.23214	0.9778	9.333	0
X_4	-16.2024	0.9778	-0.043	0.967
X^2_1	-8.26637	0.8957	-9.228	0
X^2_2	-0.30066	0.8957	-8.391	0
X^2_3	0.089658	0.8957	0.4	0.694
X^2_4	-11.5655	0.8957	-3.228	0.005
$X_1 * X_2$	1.1625	1.1975	4.854	0
$X_1 * X_3$	0.03125	1.1975	0.052	0.959
$X_1 * X_4$	-0.875	1.1975	-0.365	0.72
$X_2 * X_3$	0.29375	1.1975	2.453	0.026
$X_2 * X_4$	1.375	1.1975	2.871	0.011
$X_3 * X_4$	0.6875	1.1975	0.574	0.574

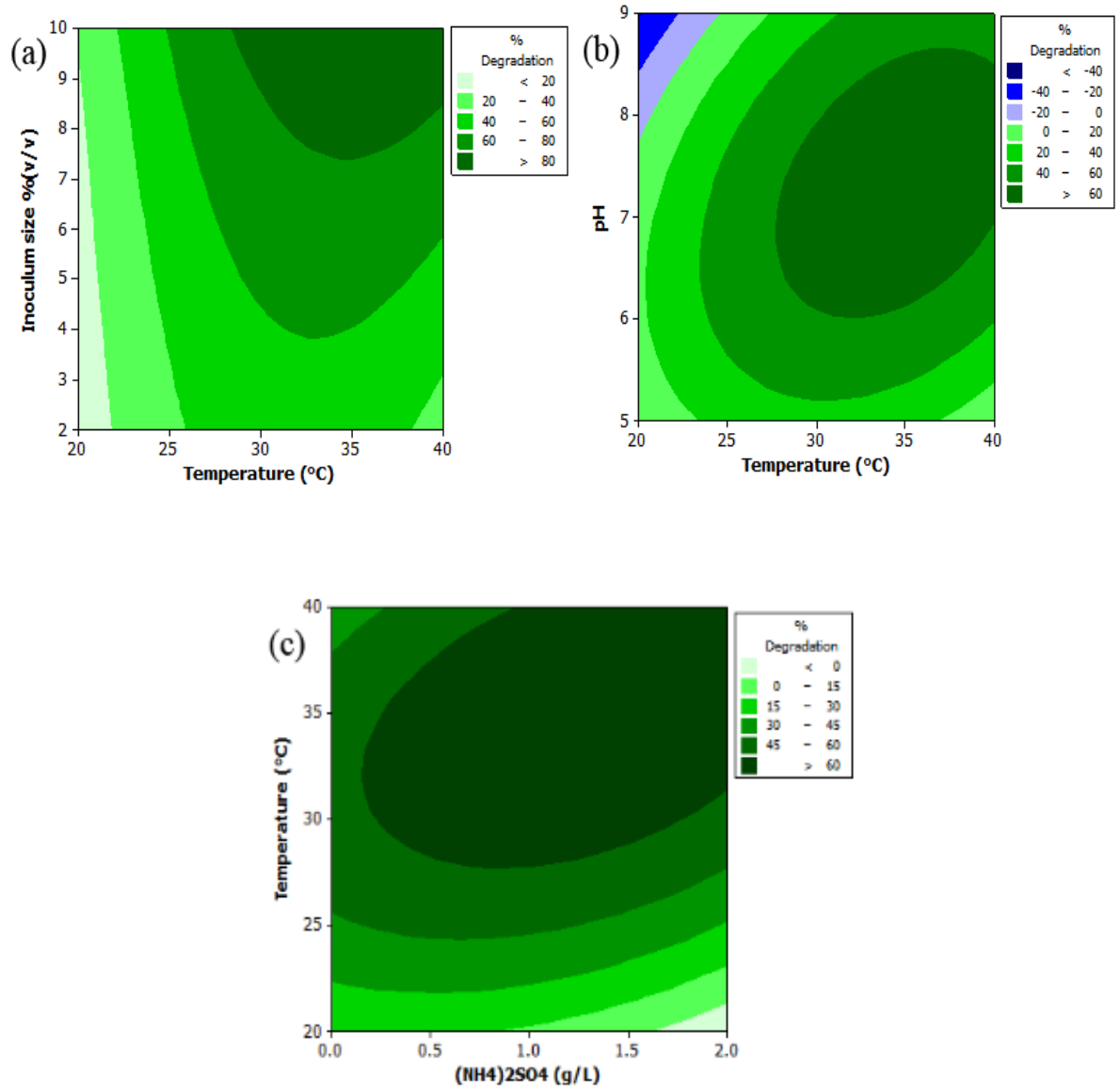


Figure 4.13: (a) Contour plot showing the interaction effect of inoculum size %(v/v) and temperature (ºC) on 2,4-DCP biodegradation. **(b)** Contour plot showing the interaction effect of temperature (ºC) and pH on 2,4-DCP biodegradation. **(c)** Contour plot showing the interaction effect of $(NH_4)_2SO_4$ (g/L) and temperature (ºC) on 2,4-DCP biodegradation.

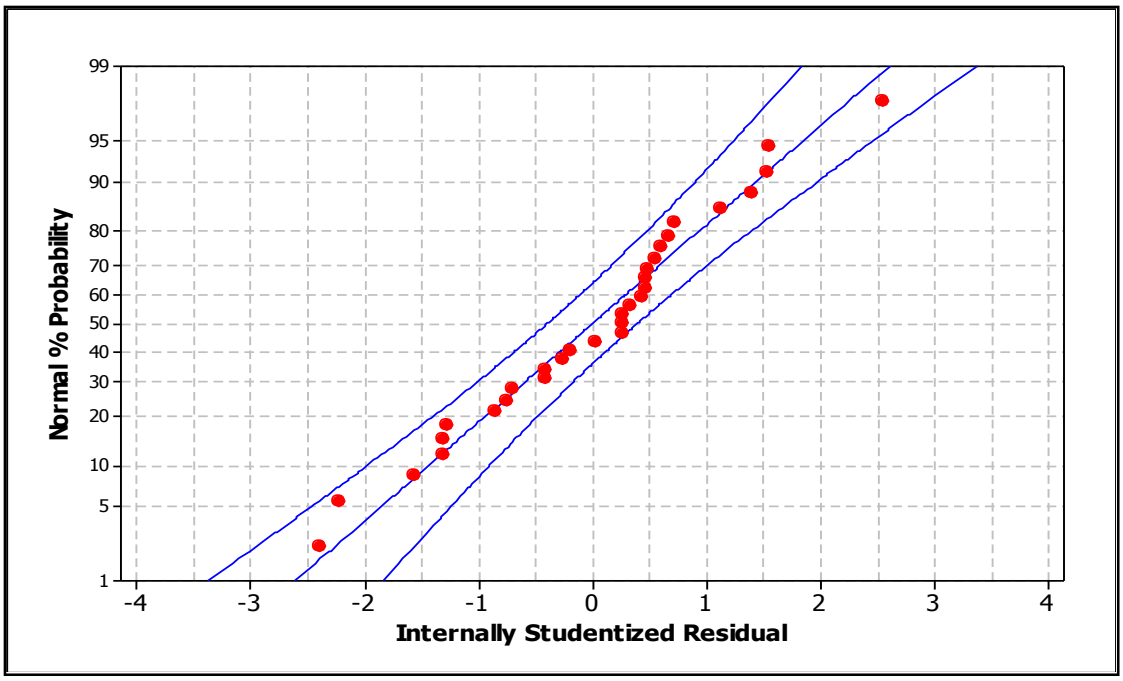


Figure 4.14: The normal probability of internally studentized residuals for % biodegradation of 2,4-DCP by *B. endophyticus* CP1R.

4.2.3 Biodegradation kinetic of 2,4-DCP at RSM optimized condition

Bacillus endophyticus CP1R strain significantly degraded 2,4-DCP up to 400 mg/L at RSM optimized conditions. The biodegradation profile and residual concentration of 2,4-DCP are shown in figure 4.15. The isolate has completely degraded 25 and 50 mg/L of 2,4-DCP whereas, more than 75–80% degradation obtained for 100–400 mg/L of initial 2,4-DCP concentration at optimized culture conditions. The relation between the 2,4-DCP removal rate and initial 2,4-DCP concentration is shown in figure 4.16. The plot is linear depicting that the 2,4-DCP removal rate increases almost linearly with initial 2,4-DCP concentration.

In this study, the higher removal rate of 2,4-DCP was observed with increasing substrate concentration. The microorganism use different bioenergetic strategies depending on the concentration and toxicity of 2,4-DCP. At lower concentration of 2,4-DCP, the microorganism directs energy to the growth instead of biodegradation leading to less 2,4-DCP removal per cell. While the microorganism directs energy to biodegradation instead of growth at higher concentration leading to higher 2,4-DCP removal per cell. Papazi and Kotzabasis also reported such observation that the degradation of high toxic dichlorophenol congener is higher as compare to low toxic dichlorophenol congener and monochlorophenol [222]. The microorganism uses a different bioenergetic strategy in such a way so that there is a balance between the 2,4-DCP biodegradability, 2,4-DCP toxicity, and growth.

The plot between the *1/Rs* vs. *1/S* was plotted for experimental data (Figure 4.17). The plot is linear with the slope of *Ks/Rm* and the intercept of *1/Rm*. From the best fit line, the following values were obtained for biokinetic parameters using Equation 3.1:

R_m = 1.53 mg DCP/L/h and K_s= 711.5 mg/L

(R^2 = **0.997**)

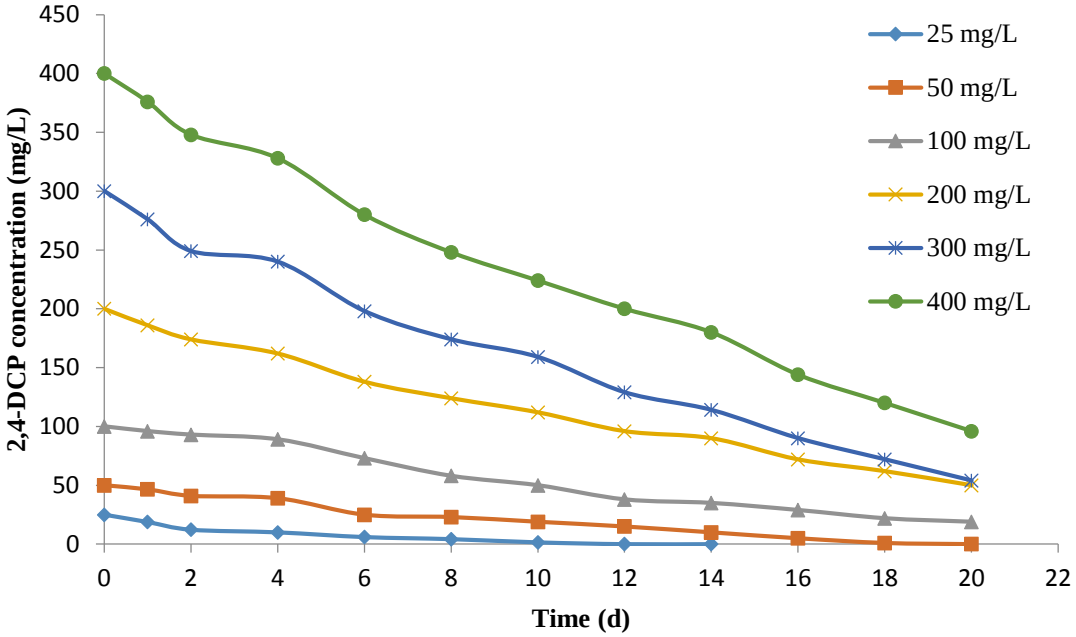


Figure 4.15: 2,4-DCP biodegradation profile at a different initial concentration by *Bacillus endophyticus* CP1R at RSM optimized conditions.

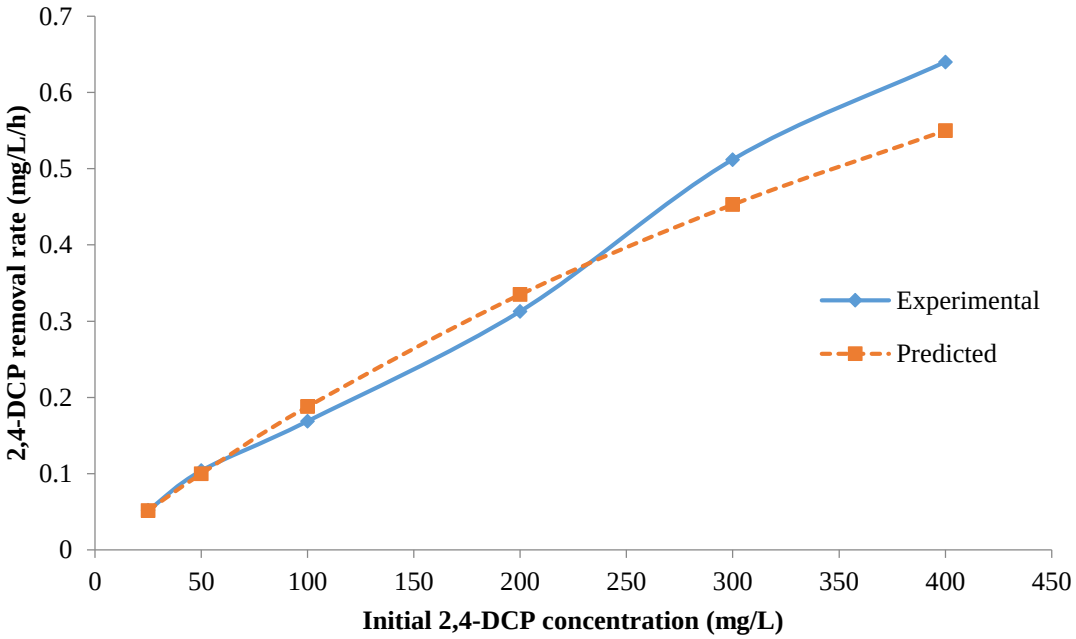


Figure 4.16: Relationship between 2,4-DCP removal rate and initial 2,4-DCP concentration at RSM optimized conditions.

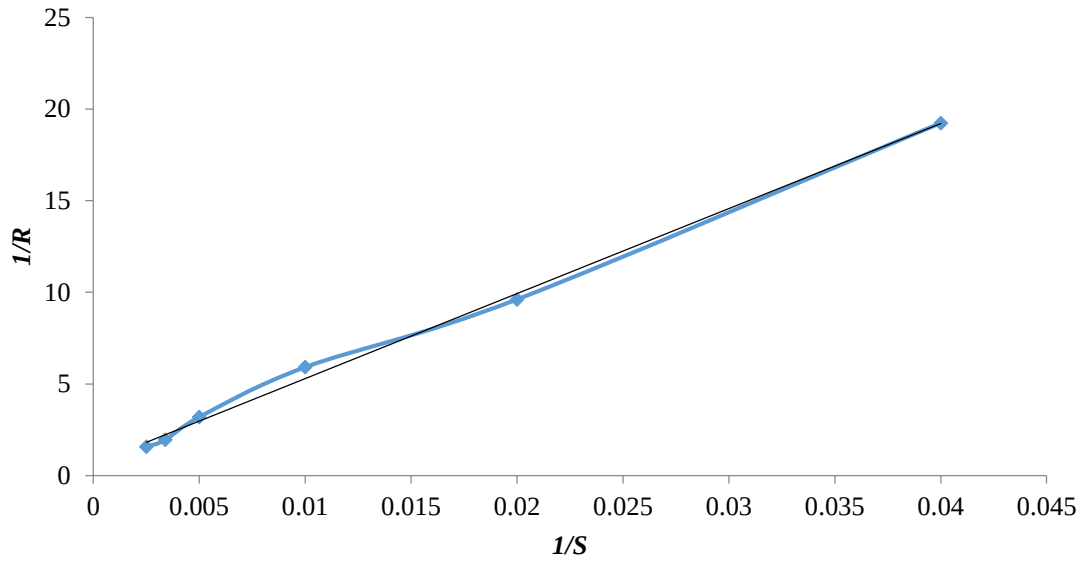


Figure 4.17: Double reciprocal plot of *1/R* v/s *1/S*.

4.2.4 Biodegradation of monochlorophenols by B. endophyticus CP1R

Biodegradation of 2-CP, 3-CP, and 4-CP by *B. endophyticus* CP1R was carried out at a different initial concentration ranging from 25 to 400 mg/L at RSM optimized conditions. In case of 2-CP, the biodegradation was carried out for 16 days. There was not abiotic loss was observed due to evaporation in case of 3-CP and 4-CP while the abiotic lossobserved for 2-CP was in the range of 2 to 17% for an initial substrate concentration of 25 to 400 mg/L. The abiotic loss was adjusted in the result presented here. The biodegradation profile of 2-CP at different initial substrate concentration has shown in figure 4.18. The strain has shown 100 and 92% degradation of 25 mg/L and 50 mg/L of 2-CP within 16 days respectively. The biodegradation rate obtained for the initial substrate concentration of 100, 200, 300 and 400 mg/L was 79, 70, 63 and 56% respectively. Figure 4.19 shows the biodegradation (%) and residual substrate concentration at different initial substrate concentration. The percent biodegradation was decreased with increasing substrate concentration. Consequently, the residual substrate concentration increases as shown in the figure 4.19. The effect of initial substrate concentration on the removal rate of 2-CP has shown in figure 4.20. From figure it can be observed that the removal rate increases almost linearly with initial 2-CP concentration indicating very low inhibition effect of 2-CP on microbes up to 400 mg/L. Among all the chlorophenols congeners, the toxicity of 2-CP is the lowest due to removal of chloride ion from *ortho* position is low energy demanding process. There was no inhibition observed for 2-CP by the strain up to 400 mg/L.

In case of 3-CP and 4-CP, the biodegradation was performed for 15 days. The strain has experienced high inhibition effect in the presence of 3-CP and 4-CP as compared to 2-CP. The strain has shown degradation up to 50 mg/L for 3-CP and 4-CP. Afterward, the inhibition effect was more persistent. The biodegradation obtained for 3-CP and 4-CP at different initial substrate concentration has shown figure 4.21. In case of 4-CP, the strain has shown 40, 25

and 12% degradation for the initial substrate concentration of 25, 50 and 100 mg/L respectively. The biodegradation rate was drastically diminished after 100 mg/L. The biodegradation obtained for 200, 300 and 400 mg/L of 4-CP was 5, 3 and 2% respectively. While in case of 3-CP, the strain has shown 74, 17 and 2% degradation for the initial substrate concentration of 25, 50 and 100 mg/L respectively. The strain was not able to utilize 3-CP after 100 mg/L of initial concentration. In both cases, the degradation rate decreases after sometimes and remains constant due to inhibition effect imposed by the accumulation of metabolites. Also, the cell death occurs due to the toxic effect imposed by the compounds and its metabolites. The effect of initial substrate concentration on the removal rate of 3-CP and 4-CP has shown in figure 4.22. The figures shows that the biodegradation of 3-CP and 4-CP follows the substrate inhibition model. Initial the removal rate for 3-CP and 4-CP increases with initial substrate concentration up to 25 mg/L and 50 mg/L respectively and afterwards it start to decreases. In case of 3-CP, the removal rate become almost zero after 100 mg/L of initial 3-CP concentration.

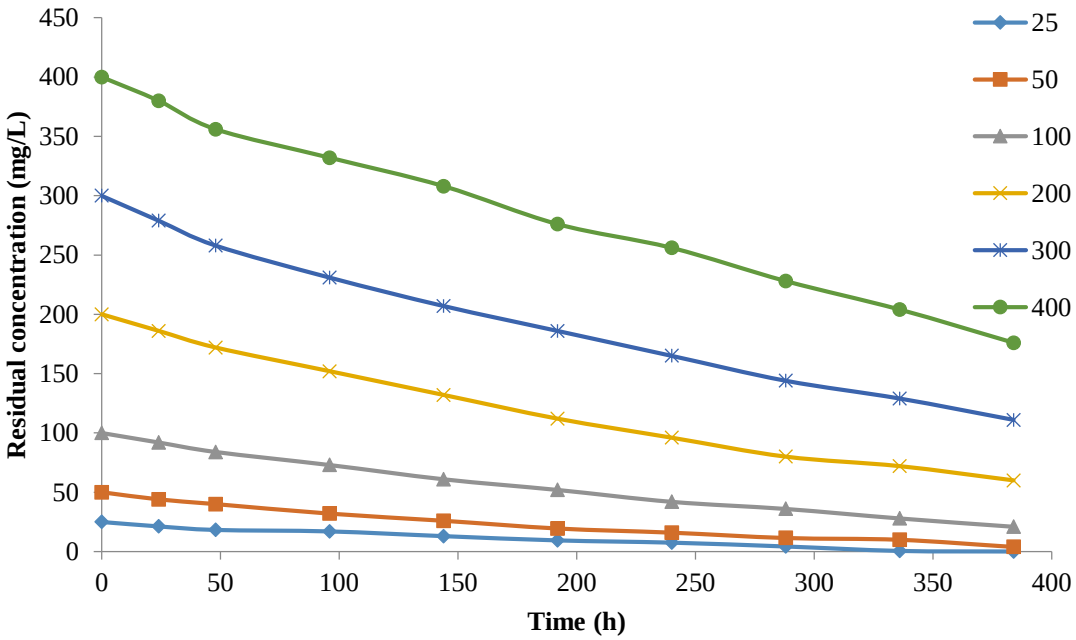


Figure 4.18: Biodegradation profile of 2-CP *B. endophyticus* CP1R at a different initial substrate concentration.

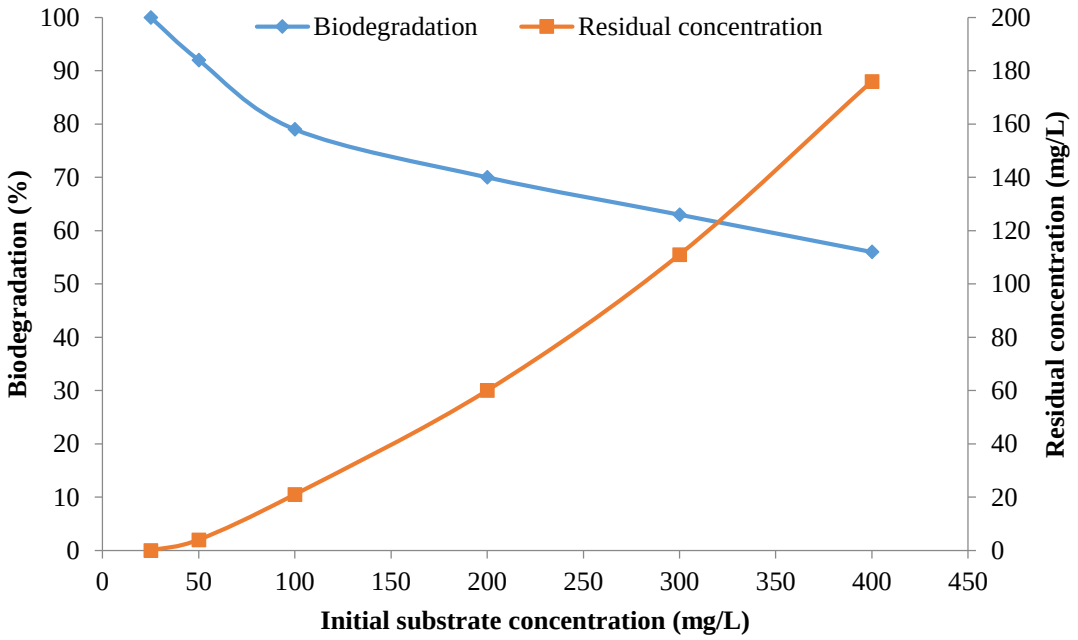


Figure 4.19: Effect of initial 2-CP concentration on biodegradation and residual concentration.

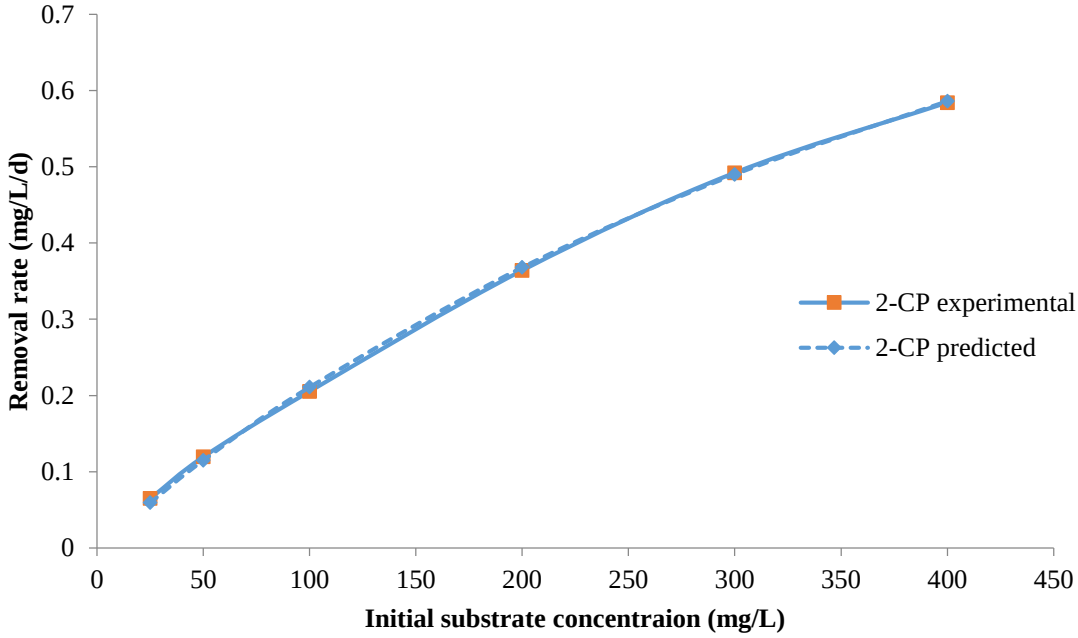


Figure 4.20: Effect of initial 2-CP concentration on the removal rate by *B. endophyticus* CP1R.

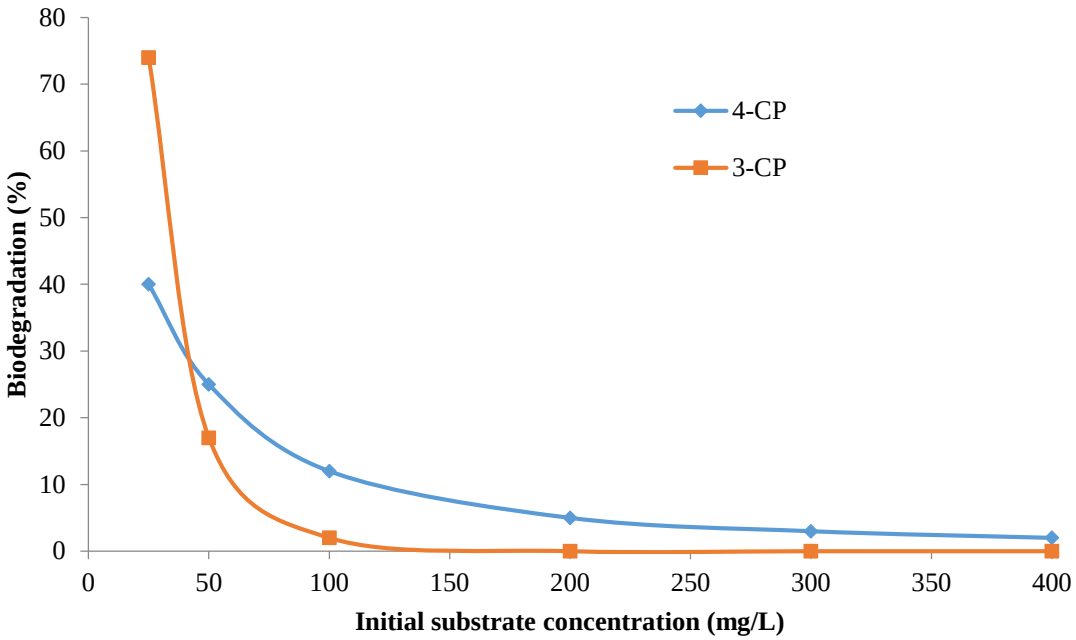


Figure 4.21: Biodegradation of 3-CP and 4-CP at different initial substrate concentration *B. endophyticus* CP1R.

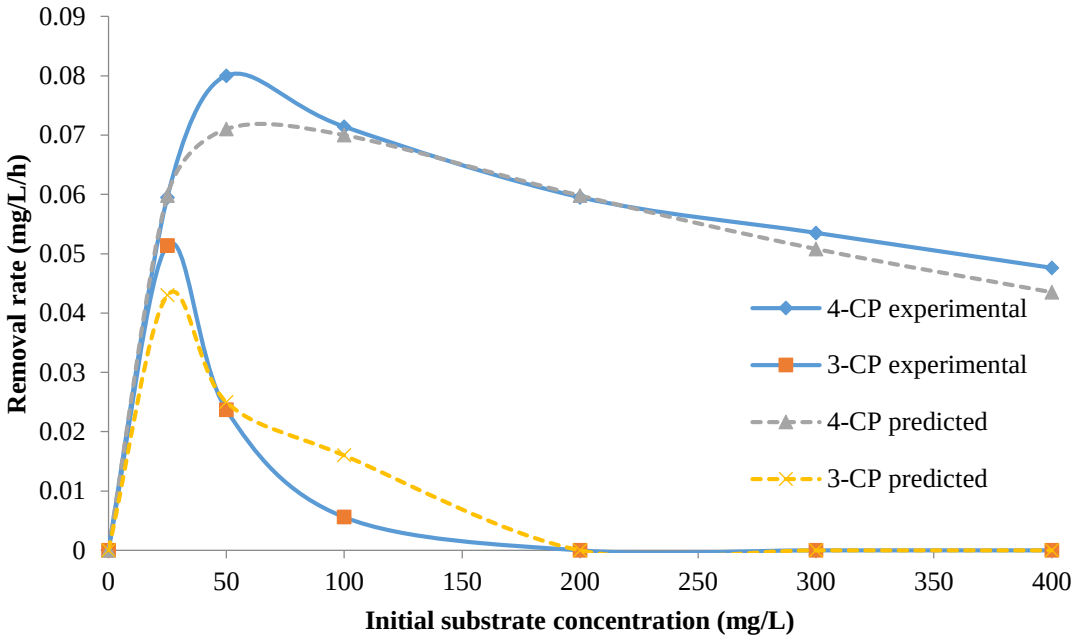


Figure 4.22: Effect of initial substrate concentration on the removal rate of 3-CP and 4-CP by *B. endophyticus* CP1R.

4.2.4.1 Biodegradation kinetic of monochlorophenols

Table 4.8 summarizes the biodegradation kinetic parameters for 2-CP, 3-CP and 4-CP degradation calculated using the Andrews substrate inhibition model (Equation 3.1). The biokinetic parameters were obtained using the GraphPad Prism and Matlab 6.5 software. The maximum removal rate obtained for 2-CP, 3-CP and 4-CP were 1.43, 0.123 and 0.118 mg/L/h respectively. In case of 2-CP, there was no inhibition effect was observed up to 400 mg/L and so the inhibition constant (K_i) can be neglected from equation 3.1. The inhibition constant obtained for 3-CP and 4-CP were 13.08 and 251.9 mg/L. The higher value of inhibition constant for 4-CP indicates the low inhibition effect on the strain as compared to 3-CP. The higher value of half saturation constant (*Ks*) for 2-CP indicates that it has reached maximum removal rate at a higher concentration. Also, it indicates the higher affinity of the strain towards 2-CP compared to 3-CP and 4-CP. While for 3-CP and 4-CP, the low value of half saturation constant indicates that the maximum removal rate was achieved at low concentration.

Table 4.8: Biodegradation kinetics parameters for monochlorophenols by *Bacillus endophyticus* CP1R using Andrews's model.

Compound	R_m **(mg/L/h)**	K_S **(mg/L)**	K_i **(mg/L)**	R^2
2-CP	1.43	575.8	-	0.999
3-CP	0.123	1	13.08	0.898
4-CP	0.118	20.18	251.9	0.982

4.2.5 Cometabolism of monochlorophenols and 2,4-dichlorophenol

The co-metabolic study is important to understand the multi-substrate degradation process that has encountered in the *in-situ* environment. Most of the co-metabolic study was done by the mixed bacterial consortium. However, there are fewer reports on the cometabolic study of 2, 4-DCP in the presence of MCPs by pure bacterial strain. In the present study, the co-metabolic study was carried out to analyze the effect of presence of MCPs (2-CP, 3-CP, and 4-CP) on biodegradation of 2,4-DCP by *B. endophyticus* CP1R. The co-metabolic study was performed in 250 mL Erlenmeyer with 50 mL MSM. Different combination of 2,4-DCP and MCPs added to the flask as mentioned in the Table 4.9. The concentration of 2,4-DCP was kept constant i.e. 100 mg/L for comparison and the concentration of monochlorophenols (2CP, 3CP & 4CP) were selected such that the total pollutant concentration in the medium remains constant i.e 150 mg/L. Overall concentrations for pollutant were selected from the previous batch study based on the tolerance and degradation level of the pollutant by the strain.

2,4-DCP has two chloride substitution, one at *ortho* and other at the *para* position. MCP has three different congeners, *ortho* (2-CP), *meta* (3-CP) and *para* (4-CP), with one

chloride substitution at a different position. The degradation of the parent compound in the presence of its metabolites was observed to decrease or increase depending on the molecular structure and enzymes expression. The 2-CP and 4-CP are degradation products of 2,4-DCP. Thus, the study of the effect of MCPs on the biodegradation of 2,4-DCP was important. The co-metabolic study of 2,4-DCP (DCP for further discussion) in the presence of three different MCPs by *B. endophyticus* CP1R was analyzed at RSM optimized condition, and the result has shown in figure 4.23 and 4.24.

Table 4.9: A different combination of 2,4-DCP and MCPs used for the co-metabolic study.

Compound	**DCP (mg/L)**	**2CP (mg/L)**	**3CP (mg/L)**	**4CP (mg/L)**	**Total CP (mg/L)**
DCP	100	-	-	-	100
DCP+2CP	100	50	-	-	150
DCP+3CP	100	-	50	-	150
DCP+4CP	100	-	-	50	150
DCP+2CP+3CP+4CP	100	25	12.5	12.5	150

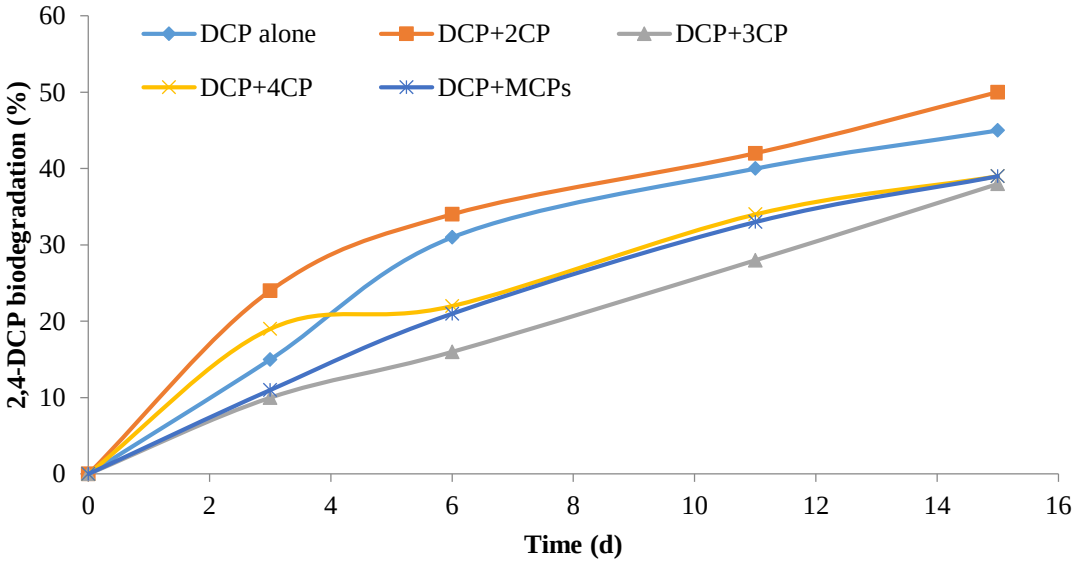


Figure 4.23: Biodegradation of 2,4-DCP by *B. endophyticus* CP1R in the presence of monochlorophenols.

The strain has achieved 45% degradation of DCP alone for 100 mg/L of initial concentration. In the binary mixture of DCP and MCPs, the biodegradation observed for DCP was 50, 38 and 39% in the presence of 2-CP, 3-CP and 4-CP respectively. While the biodegradation observed for 2-CP, 3-CP and 4-CP were 95, 0 and 0% respectively. The strain was not able to degrade 3-CP and 4-CP in the presence of DCP. The biodegradation of DCP

was increased in the presence of 2-CP as compared to that obtained for DCP alone. However, the biodegradation was decreased in the presence of 3-CP and 4-CP. The biodegradation in the binary mixture was observed in the order of 2-CP>DCP>3-CP≈4-CP.

In the quaternary mixture, the biodegradation obtained for DCP was 39% that was lower than that obtained for DCP alone and in a binary mixture. While the biodegradation observed for 2-CP, 3-CP and 4-CP were 36, 0 and 0% respectively. The biodegradation in the quaternary mixture was observed in the order of DCP>2-CP>3-CP≈4-CP.

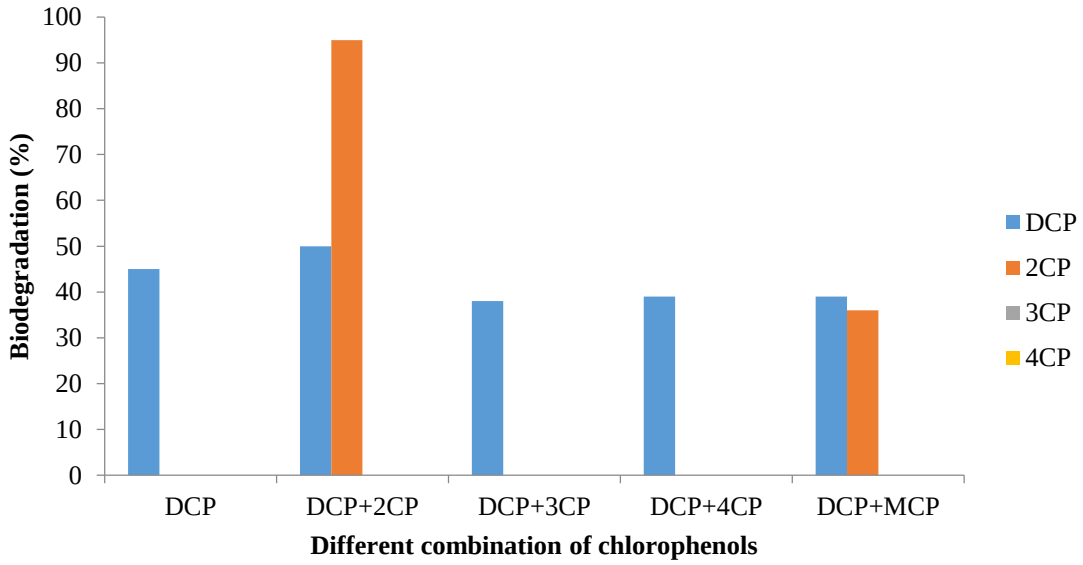


Figure 4.24: Biodegradation (%) of different chlorophenols during co-metabolic study by *B. endophyticus* CP1R.

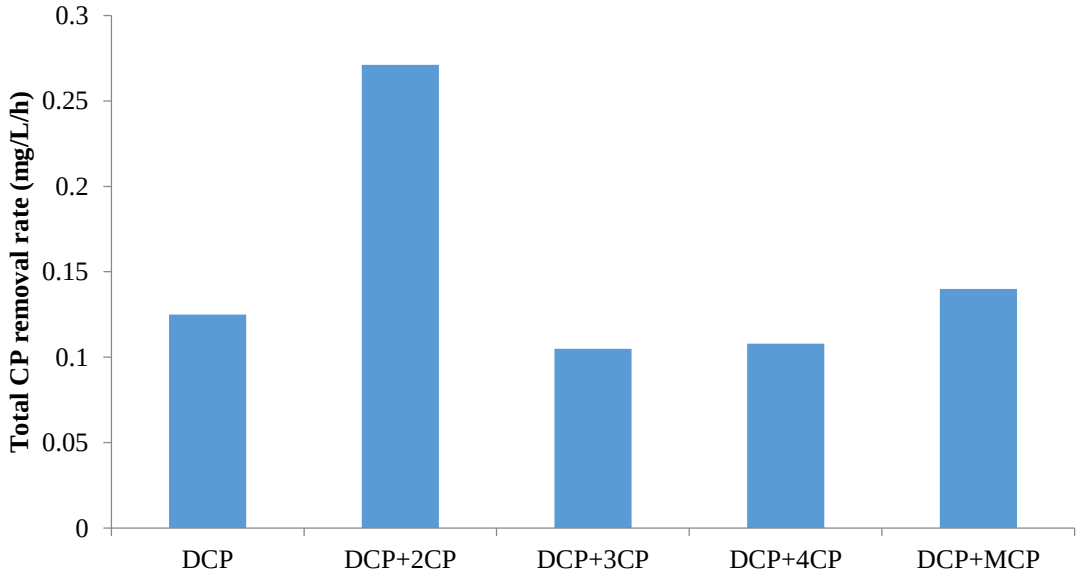


Figure 4.25: Total chlorophenol removal rate for the different combination of chlorophenols by *B. endophyticus* CP1R.

The total chlorophenol removal rate obtained for the different combination of MCPs and DCP has shown in figure 4.25. The highest chlorophenol removal rate obtained was 0.271 mg/L/h for a binary mixture of 2-CP and DCP. The presence of 2-CP enhances the biodegradation of DCP compared to 3-CP and 4-CP. The toxicity of chlorophenols are in the order of DCP>3-CP>4-CP>2-CP. The combination of DCP with 3-CP and 4-CP is more toxic than with 2-CP. There were reports that show the presence of low toxic compounds increases the biodegradation of high toxic compounds.

In a binary mixture, DCP has a higher toxicity than 3-CP and 4-CP. But the strain has only shown degradation of DCP (higher toxic) and not 3-CP and 4-CP (low toxic). In the quaternary mixture also, the degradation of DCP is higher than the corresponding MCPs. The actual reason or mechanism involved was not known. The enzymes involved were only able to dechlorinate or attack at the *ortho* position. The *para* and *meta* position shows more electron hindrance than the *ortho* position. Similar results have also been reported, in which higher toxic compounds were observed to be degraded faster compared to the relatively lower toxic compounds. Zilouei et al., (2006) [24] reported the biodegradation of 2,4-DCP and 2,4,6-TCP to be higher as compared to 4-CP and 2-CP by the mixed consortium. The order of removal increases as TCP>DCP>4CP>2CP. The easily degradable 2-CP was removed at the slowest rate. However, the exact mechanism for this phenomenon has not been reported by the authors. Another study also reported similar findings during the cometabolism of MCPs by *P. aeruginosa* strain. The *Pseudomonas* strain was able to utilize 3-CP and 4-CP, both singly and in the mixture up to 25 mg/L, while the strain was unable to utilize 2-CP in the mixture [223]. Farrell and Quilty (1999) [40] have also reported the degradation of MCPs by the mixed consortium in which the degradation of 4-CP and 3-CP were reported to be higher as compared to 2-CP. Papazi and Kotzabasis (2013) have reported that the microorganisms utilize different bio-energetic processes for degradation of compounds depending on their toxicity. In the presence of higher toxic compounds or when toxicity of compounds reaches a threshold level, the microorganism gives more energy to toxicity removal or biodegradation than to biomass growth. They have also reported that the biodegradation of DCP congeners 2,3-DCP, 2,5-DCP and 3,4-DCP (higher toxicity, one *meta* substitution) was higher compared to DCP congeners 2,4-DCP and 2,6-DCP (lower toxicity, no *meta* substitution) and the corresponding MCPs [224]. Cometabolic study of 2,4-DCP with three monochlorophenols shows that the isolate has able to utilize 2,4-DCP in the presence of all the MCPs. This shows the great prospect of the strain for *in-situ* bioremediation purpose.

4.3. Biodegradation of chlorophenols by *Kocuria rhizophila* strain 11Y: Optimization, Kinetic and Cometabolism

In this section, the biodegradation of six different chlorophenols including 2-CP, 3-CP, 4-CP, 2,4-DCP, 2,4,6-TCP and PCP by ***Kocuria rhizophila* strain 11Y** were studied. The growth and biodegradation kinetics of the chlorophenols biodegradation were studied. The optimization of four different environmental parameters was performed using the response surface methodology to achieve the maximum biodegradation of 2,4-DCP. At last, the cometabolism of 2,4-DCP in the presence of all the three monochlorophenols by the isolate were evaluated. The isolate hasn't shown any degradation for 2,4,6-TCP and PCP while it has shown only negligible degradation for 3-CP and 4-CP. However, the strain has shown great removal efficiency of 2-CP and 2,4-DCP.

4.3.1 Biodegradation and kinetic of 2,4-DCP by strain 11Y

Biodegradation of 2,4-DCP by *Kocuria rhizophila* strain was carried out in batch mode for 312 h. The residual concentration of 2,4-DCP and biodegradation profile has shown in figure-4.26. The percent biodegradation decreases and residual concentration increases with increase in initial 2,4-DCP concentration. The strain was able to degrade 25 mg/L of 2,4-DCP completely after that degradation rate decreases. The strain has shown 75% degradation of initial 2,4-DCP concentration of 100 mg/L and the degradation was decreased to 67 and 47% for 200 and 300 mg/L of initial 2,4-DCP concentration respectively. Figure 4.27 shows the effect of initial 2,4-DCP concentration on the removal rate. The 2,4-DCP removal rate increases with initial 2,4-DCP concentration up to 200 mg/L after that the rate remains constant. This may be due to the inhibition effect prevail after 200 mg/L of 2,4-DCP concentration. The overall maximum biodegradation rate observed was 0.61 mg DCP/h/L (Equation 3.2). The pH drop was observed from 7.0 to 6.5-6.8 due the release of chloride ion in the medium during biodegradation.

Biodegradation of 2,4-DCP in the medium was confirmed by HPLC and mass spectroscopy. The sample was acidified with 1M HCl and extracted with ethyl acetate. The extracted sample was analyzed using electrospray ionization mass spectroscopy for metabolites. Various metabolites formation was observed in ethyl acetate extract of biodegradation sample of 2,4-DCP (400 mg/L) by ESI-MS data (figure 4.28). The highest peak (m/z=370) might related to the conjugation product of two sodium 2,4-dichlorophenoxy ion [225]. The m/z peak 177, 178, 179, 199, 200, and 79 was corresponding to 3,5-dichlorocatechol [218]. The multiplets (152, 129, 111, and 101) were identified as monochlorophenol and its conjugate fragments. Other m/z peaks (354, 242, 223, and 180) related to the degradation product or conjugate fragment were also detected.

4.3.1.1. Effect of glucose on 2,4-DCP degradation

In this study, the different initial concentration of glucose (0.5, 1, 1.5, 2 g/L) was added to the MSM containing 50 mg/L 2,4-DCP. The percent 2,4-DCP degradation observed for different initial glucose concentration was shown in figure 4.29. The rate of biodegradation significantly increases with increase in the initial concentration of glucose.

The maximum 99% degradation of 50 mg/L 2,4-DCP was observed within 96 h for 2 g/L glucose. The rate of biodegradation decreases to 87, 64 and 47% for 1.5, 1 and 0.5 g/L initial glucose concentration respectively. Without glucose, the strain was able to degrade only 85% of 50 mg/L of 2,4-DCP within 312 h.

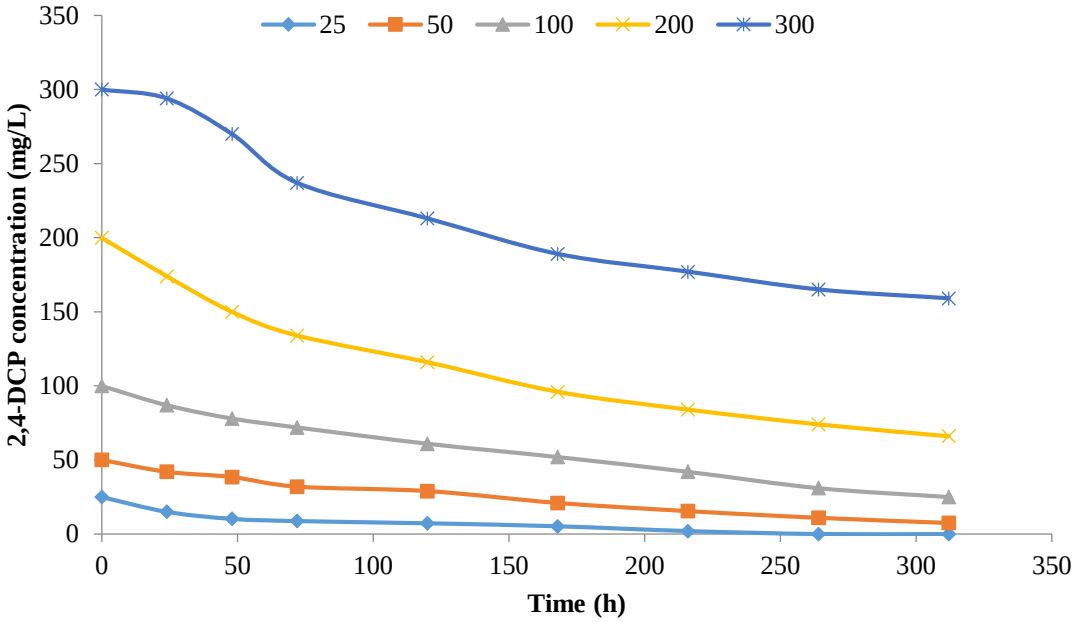


Figure 4.26: Biodegradation profile of 2,4-DCP by *Kocuria rhizophila* 11Y at different initial substrate concentration.

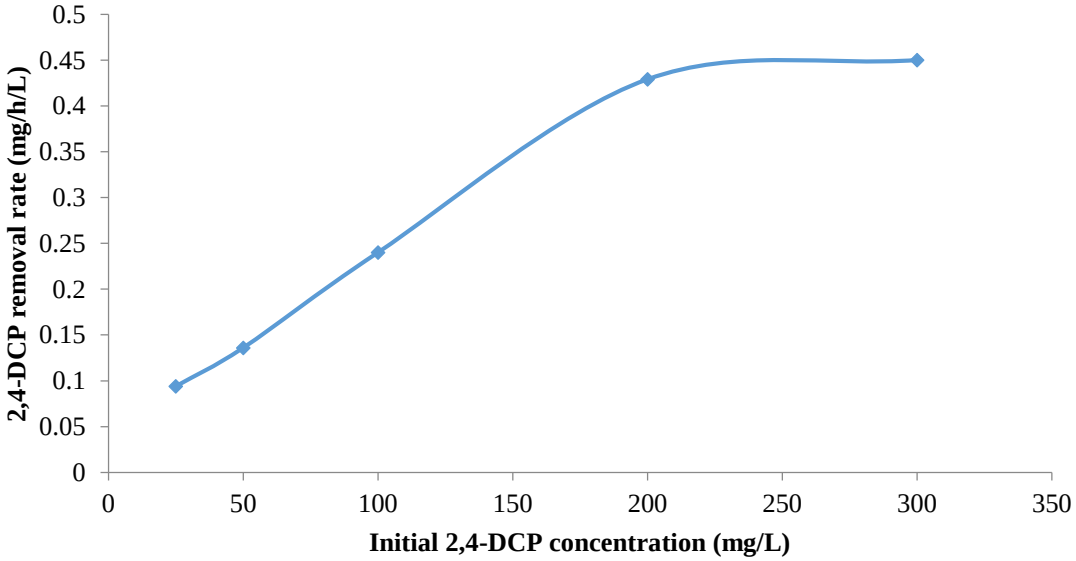


Figure 4.27: Effect of initial 2,4-DCP concentration in the removal rate by *Kocuria rhizophila* 11Y.

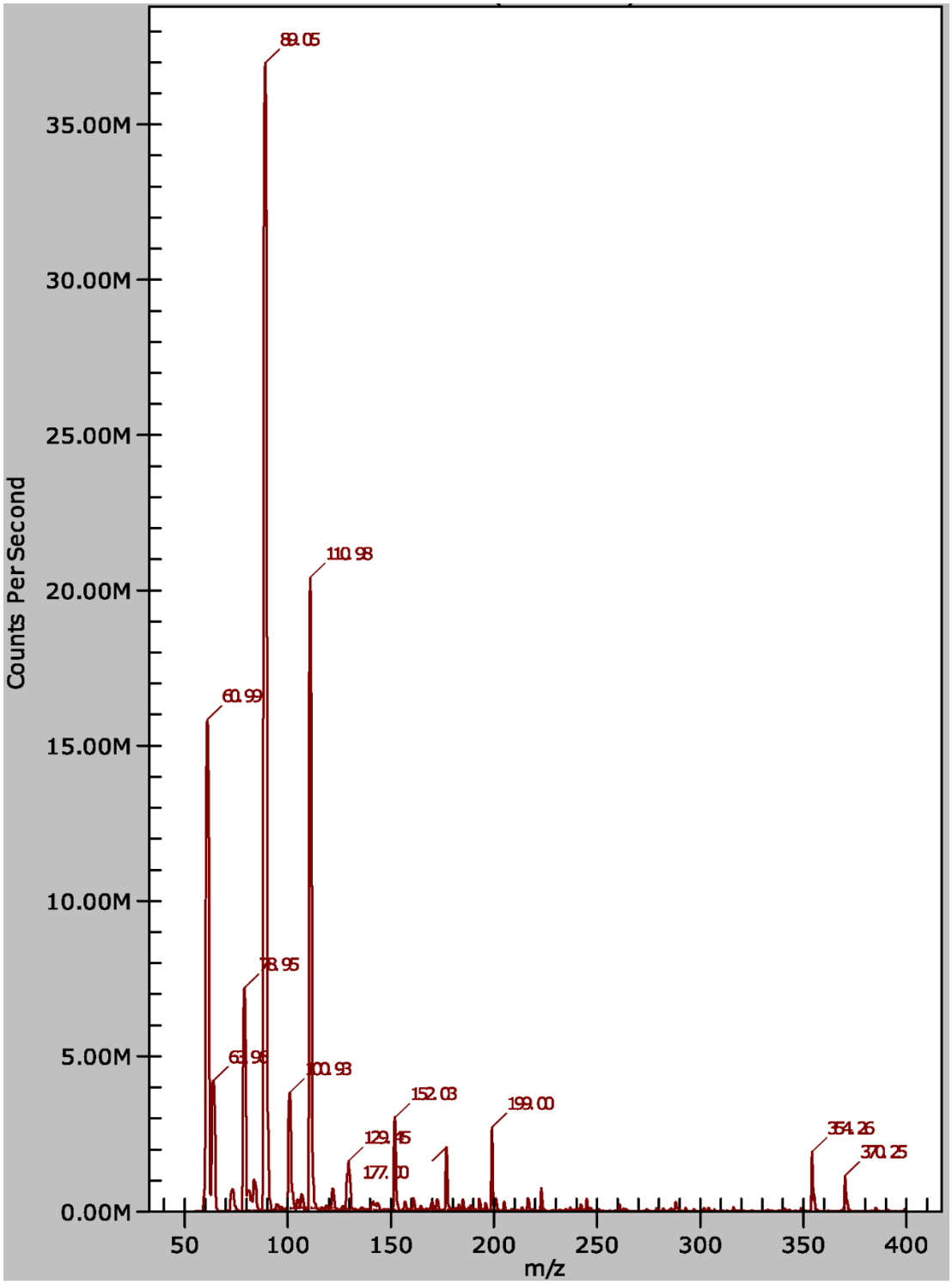


Figure 4.28: Mass spectrum of 2,4-DCP (400 mg/L) biodegradation products by *K. rhizophila* 11Y.

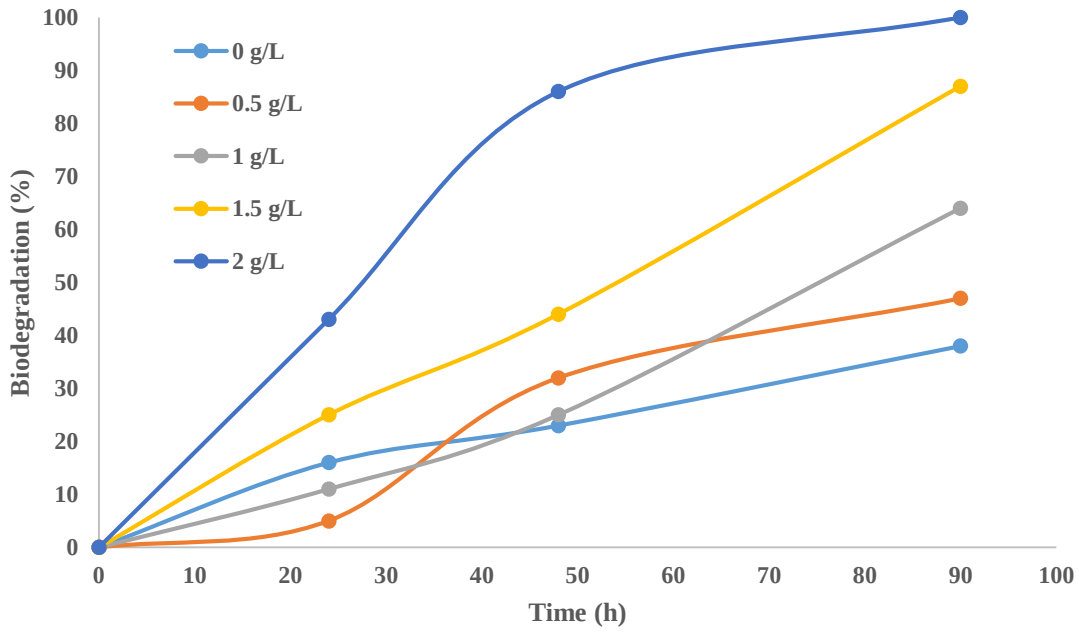


Figure 4.29: Effect of glucose concentration on biodegradation profile of 2,4-DCP by *Kocuria rhizophila* 11Y.

4.3.1.2. Growth kinetic study:

The plot showing the relationship between the specific growth rate and initial substrate concentration has shown in figure 4.30. The 2,4-DCP degradation by *Kocuria rhizophila* strain follows the substrate inhibition kinetic as the specific growth rate decreases with increase in initial substrate concentration. The Haldane substrate inhibition model was fittedto experimental data using Matlab 6.5 and the biokinetic parameters obtained were: maximum specific growth rate (μ_m) = 0.307 h^{-1}, half saturation constant (K_s) = 286.7 mg/L and substrate inhibition constant (K_i) = 4.9 mg/L.

$$\mu = \frac{0.307\, s}{s + 286.7 + \frac{s^2}{4.9}}$$

The biodegradation of 2,4-DCP by *Kocuria rhizophila* has not been reported in the literature to the best of our knowledge. So the comparison of the obtained data with other *Kocuria* sp. was not possible. Kargi and Eker (2005) reported the degradation of 50 to 750 mg/L of 2,4-DCPby *Pseudomonas putida* CP1. The percentage 2,4-DCP removal was declined from 35% at the lowest initial 2,4-DCP concentration of 51mg/L to 13% at the highest initial 2,4-DCP concentration of 758mg/L. The maximum rate of 2,4-DCP degradation was found 0.8 mg DCP/L/h at an initial 577 mg DCP/L and declined for higher initial DCP concentration [184]. In another study by *Pseudomonas putida* (DSM 6978), the degradation of 2,4-DCP was found to be 100, 26, and 12% for the initial 2,4-DCP concentration of 30, 160, and 300 mg/L respectively [220]. The degradation of 2,4-DCP by

Pseudomonas sp. was found in the range of 11 to 65% for initial 2,4-DCP concentration of 0.1 to 1 mM in the presence of 0.1% glucose [207].

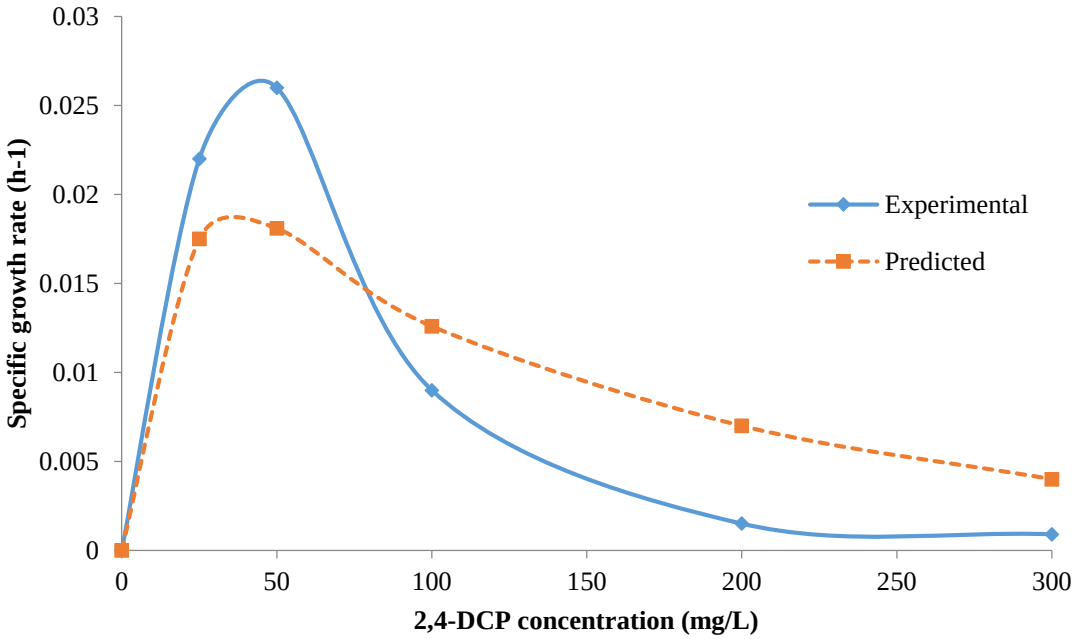


Figure 4.30: Effect of initial 2,4-DCP concentration on the specific growth rate of *Kocuria rhizophila* 11Y.

4.3.2 Optimization of experimental parameters to achieve maximum biodegradation of 2,4-DCP by stain 11Y.

The optimization of various experimental parameters i.e. pH, temperature (ºC), Inoculum size %(v/v) and ammonium sulphate concentration (g/L) at different levels were carried out to enhance the 2,4-DCP degradation by *Kocuria rhizophila* strain 11Y. The biodegradation kinetic parameters for 2,4-DCP were also calculated for the same at optimized condition. The central composite design of RSM was used to optimize the parameters. The independent variables and their corresponding levels used in the study have shown in Table 4.10.

4.3.2.1 Optimization of experimental parameters using RSM

The optimization of experimental parameters for achieving maximum biodegradation of 2,4-DCP by *Kocuria rhizophila* strain 11Y has successfully performed using response surface methodology. The effect of all four independent variables i.e. pH, temperature (°C), inoculum size %(v/v) and $(NH_4)_2SO_4$ (g/L) at different levels was simultaneously analyzed on 2,4-DCP biodegradation. The experimental condition and corresponding percent biodegradation obtained as per central composite design was summarized in Table 4.11. The experimental data was analyzed in terms of the second order polynomial equation and ANOVA.

Table 4.10: Independent variables and their corresponding levels used in the optimization study for *K. rhizophila* 11Y

Factors	Coded Unit	-α	-1	0	+1	+α
pH	X_1	5	6	7	8	9
Temperature (°C)	X_2	20	25	30	35	40
Inoculum Size %(v/v)	X_3	2	4	6	8	10
$(NH_4)_2SO_4$ (g/L)	X_4	0	0.5	1	1.5	2

[a] one mL inoculum equals to ~12.78 mg dry biomass.

Figure 4.31 show the relationship between the experimental and predicted 2,4-DCP biodegradation. The coefficient of determination (R^2) and adjacent (R^2) obtained was 0.933 and 0.874 which are closer to 1, which indicates that the model is sufficiently significant and fit the data. The analysis of variance (ANOVA) and the regression coefficient for 2,4-DCP degradation was summarized in Table 4.12 and 4.13 respectively. The *f*-value and *p-value* were used to test the significance of the regression model. Higher *f*-value means that the model significantly explains the relation between the dependent and independent variables. The observed *f*-value for regression model is 15.94 which is higher than critical *f*-value ($f_{0.05,14,16}$= 2.33) at a significant level of p = 0.05 indicating that the regression model is significant and explains all the variation. The observed *f*-value for linear and square terms is greater than critical $f_{0.05,4,16}$= 3 which implies their significant role in the regression model for 2,4-DCP biodegradation. While in case of interaction terms, the critical $f_{0.05,6,16}$= 2.74 is higher than the calculated f = 1.26 implying their insignificance in the regression model.

The ANOVA table summarizes the linear, squared and interaction terms. The small *p*-value for linear and square terms indicates their significant contribution to the regression model. The *p*-value for interaction terms is 0.327 > 0.05 indicating their insignificant effect on the regression model. From the Table 4.13, it was concluded that the main effect of temperature, inoculum size and ammonium sulfate concentration are significant at an individual significant level of p = 0.05. The small *p*-value (p<0.05) for quadratic terms pH × pH, temperature × temperature and inoculum size × inoculum size and also for the interaction terms pH × pH indicate their effects are statistically significant. Thus, the regression model equation (in uncoded form) showing the effect of all four independent variable including interaction effect on 2,4-DCP biodegradation can be presented as below:

$$Y = -216.64 + 2.34X_2 - 9.2X_3 + 8.04\,X_4 - 6.12X_1^2 - 0.08X_2^2 + 0.72X_3^2 + 0.73X_1X_2$$

Where, Y = 2,4-DCP biodegradation, X_1 is pH, X_2 is temperature (°C), X_3 is inoculum size %(v/v) and X_4 is $(NH_4)_2SO_4$ (g/L).

The interaction effect between independent variables on response was well illustrated by using the contour plot and response surface. The interaction effect of the pH and

temperature are illustrated using contour plot in figure 4.32(a) while keeping other two factors at middle setting. The contour plot is elliptical shape showing the significant interaction between the factors. From the plot, it was seen that the biodegradation (%) increases with temperature and maximum biodegradation obtained in the range of 35 to 40 ºC whereas the optimum pH condition was around 7.5. However, it was observed that the bacterial growth rate decreases at 40 ºC. Figure 4.32(b) shows the effect of interaction between the pH and $(NH_4)2SO_4$concentration while keeping other two factors at middle setting, on biodegradation. From the shape of the contourplot, it could be observed that the effect of pH is more significant than $(NH_4)_2SO_4$ concentration on the response. Also, the biodegradation (%) increases with $(NH_4)_2SO_4$ concentration. By using the middle point setting the optimum values for maximum the 2,4-DCP biodegradation is around 7.3 to 7.5 for pH and is in the range of 1.5 to 2 g L^{-1} for $(NH_4)_2SO_4$ concentration. The interaction effect between the other factors appears not significant to the response. The effect of inoculum size and pH on biodegradation (%), while keeping other factors at middle setting, was shown in figure 4.32(c). From the figure, it can be seen that a higher inoculum size has pronounced effect on biodegradation. This effect diminishes with a decrease in inoculum size. The internally studentized residuals were analyzed to check the model adequacy. The analysis shows that all the studentized residuals, except four, have values under 2. The normality plot of residuals shows that all the residuals fall along the straight line (Figure 4.33).

Using the desirability function, the optimum values of experimental parameters obtained were: pH 7.45, temperature 36 °C, inoculum size 10 %(v/v) and $(NH_4)_2SO_4$ concentration 1.6 g/L. These optimum values were verified experimentally in batch mode using shake flask culture. The maximum of 98% degradation for 50 mg/L of 2,4-DCP within 20 days was observed which is 15.2% higher than at un-optimized conditions. Also, the strain was able the degrade high concentration of 2,4-DCP up to 400 mg/L at optimized conditions which is much higher than at un-optimized conditions. The strain was able to degrade up to 300 mg/L of 2,4-DCP at un-optimized conditions after that inhibition effect used to prevail.

There are several reports on optimization of different environmental parameters for improvising biodegradation of phenolic compounds using response surface methodology. RSM proves effective in terms of economic aspect, time and resource utilization for optimization process compare to traditional methods. The ammonium sulfate is important as it provide nitrogen source that is significant for bacterial growth and expression of different enzymes. Ammonium sulfate is cheap and easily available nitrogen source as compared to another nitrogen source such as amino acids, yeast extract, and peptone, etc. Optimum nitrogen concentration is significant for the microorganism to show maximum enzyme activity. The effect of ammonium sulfate as nitrogen source on biodegradation of various toxic compounds such as phenol, 2,4-DCP and also on enzyme expression has also been reported [173, 226, 227]. Temperature has an equivalent role as nutrient on microorganism activity and biodegradation. In the present study optimum temperature obtained was 36 ºC. However, the isolate has shown activity up to 40 ºC temperature but there was no significant increase in growth and degradation efficiency observed. Most of the mesophilic microorganism reported in the literature has an optimum temperature range from 28 to 35 ºC

for biodegradation [165, 169, 226]. The optimum pH obtained was 7.45 which are in good agreement with literature. At acidic pH, the toxicity of chlorophenols is higher because at this condition chlorophenols are generally present in their unionized states that readily absorbed to skin and lipid membrane leading to decreased biomass growth and biodegradation [32, 169, 228]. The pH above 7.45 has an adverse effect on enzymes leading to decreased biodegradation of 2,4-DCP. The optimum pH for maximum removal of chlorophenols reported in the literature was in the range of 7.0 to 7.5 [165, 169]. The inoculum size or biomass concentration also has an effect on biodegradation of chlorophenol compounds. With increasing inoculum size, biodegradation rate also increases. However, it was also reported that after certain inoculum size, an increase in inoculum size does not have a significant effect on growth and biodegradation due to limited nutrient condition [170, 171].

Table 4.11: Central composite design of experiments and % biodegradation of 2,4-DCP for *K. rhizophila* 11Y

Run Order	pH	Temperature (°C)	Inoculum Size %(v/v)	$(NH_4)_2SO_4$ (g/L)	% Degradation	
					Experimental	Predicted
1	0	0	0	0	62	60.14
2	0	2	0	0	70	71
3	0	0	0	0	59	60.14
4	0	0	2	0	90	93.66
5	2	0	0	0	35	38.5
6	0	0	0	0	59	60.14
7	0	0	-2	0	42	49.66
8	1	-1	-1	1	34	33.58
9	0	0	0	0	60	60.14
10	0	-2	0	0	21	31.33
11	-1	-1	1	-1	56	51.41
12	-1	-1	1	1	62	60.25
13	1	1	1	-1	78	77.08
14	1	-1	1	-1	52	49.75
15	1	1	-1	-1	55	52.58
16	1	1	-1	1	63	60.41
17	-1	1	-1	1	55	53.08
18	1	-1	-1	-1	31	25.25
19	0	0	0	0	61	60.14
20	1	1	1	1	85	86.41
21	0	0	0	2	63	66.5
22	0	0	0	0	61	60.14
23	0	0	0	0	58	60.14
24	-1	1	1	-1	68	64.25
25	-1	1	1	1	74	72.58

26	-2	0	0	0	25	32.83
27	-1	1	-1	-1	48	46.25
28	1	-1	1	1	65	59.58
29	-1	-1	-1	1	47	40.75
30	0	0	0	-2	42	49.83
31	-1	-1	-1	-1	39	33.41

Table 4.12: Analysis of Variance for % biodegradation of 2,4-DCP by *K. rhizophila* 11Y

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Regression	14	7525.55	7525.55	537.54	15.94	0
Linear	4	5729	5729	1432.25	42.46	0
Square	4	1540.55	1540.55	385.14	11.42	0
Interaction	6	256	256	42.67	1.26	0.327
Residual Error	16	539.69	539.69	33.73		
Lack-of-Fit	10	527.33	527.33	52.73	25.6	0
Pure Error	6	12.36	12.36	2.06		
Total	30	8065.24				

R-Sq = 93.31% ; R-Sq(adj) = 87.45%

DF- degree of freedom; Seq SS- sequential sum of squares; Adj MS- adjusted means square

Table 4.13: Regression coefficient for 2,4-DCP biodegradation by *K. rhizophila* 11Y

Term	Coefficient	SE Coefficient	T	*p*
Constant	-216.649	2.195	27.398	0
X_1	59.9583	1.186	1.195	0.25
X_2	2.34405	1.186	8.365	0
X_3	-9.20536	1.186	9.279	0
X_4	8.03571	1.186	3.515	0.003
X^2_1	-6.11905	1.086	-5.634	0
X^2_2	-0.08976	1.086	-2.066	0.05

X^2_3	0.720238	1.086	2.653	0.017
X^2_4	-1.97619	1.086	-0.455	0.655
$X_1 * X_2$	0.725	1.452	2.497	0.024
$X_1 * X_3$	0.8125	1.452	1.119	0.28
$X_1 * X_4$	0.5	1.452	0.172	0.865
$X_2 * X_3$	-1.14E-16	1.452	0	1
$X_2 * X_4$	-0.05	1.452	-0.086	0.932
$X_3 * X_4$	0.375	1.452	0.258	0.799

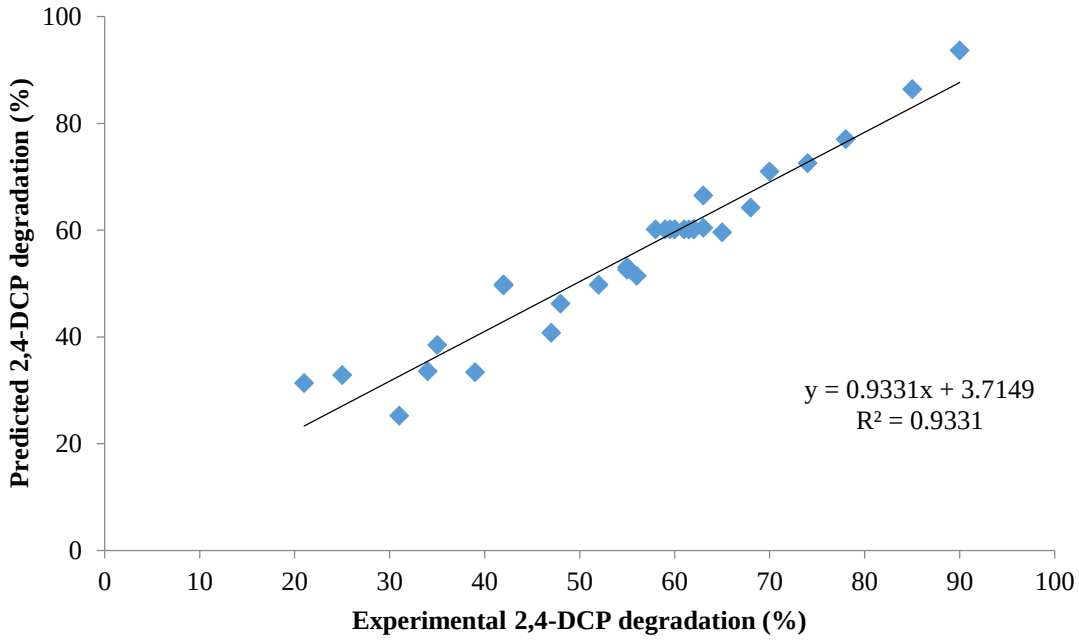


Figure 4.31: The Linear plot for experimental versus predicted biodegradation of 2,4-dichlorphenol.

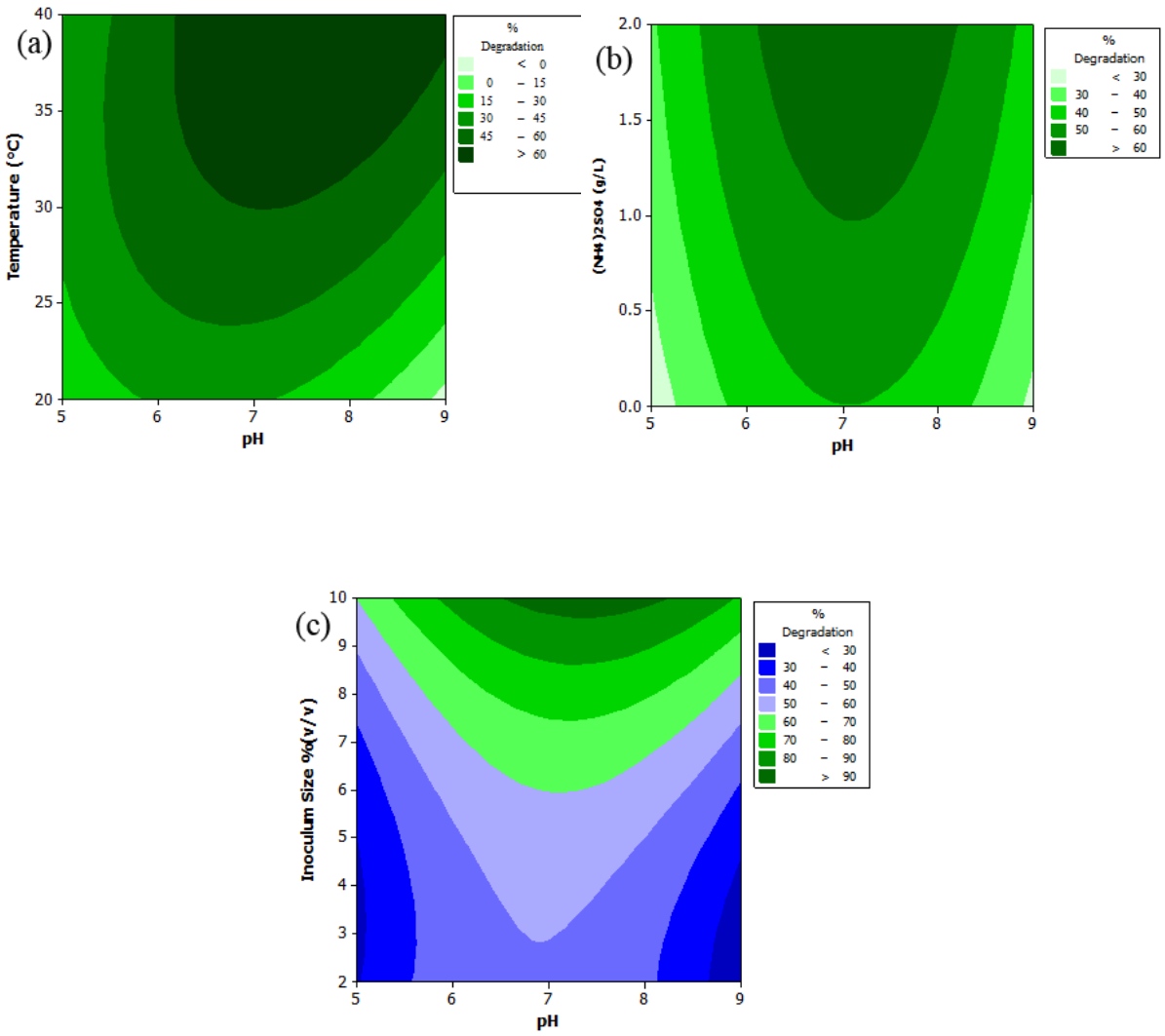


Figure 4.32: (a) Contour plot showing the interaction effect of pH and temperature (ºC) on 2,4-DCP biodegradation **(b)** Contour plot showing the interaction effect of pH and $(NH_4)_2SO_4$ (g/L) on 2,4-DCP biodegradation **(c)** Contour plot showing the interaction effect of pHand Inoculum size %(v/v) on 2,4-DCP biodegradation.

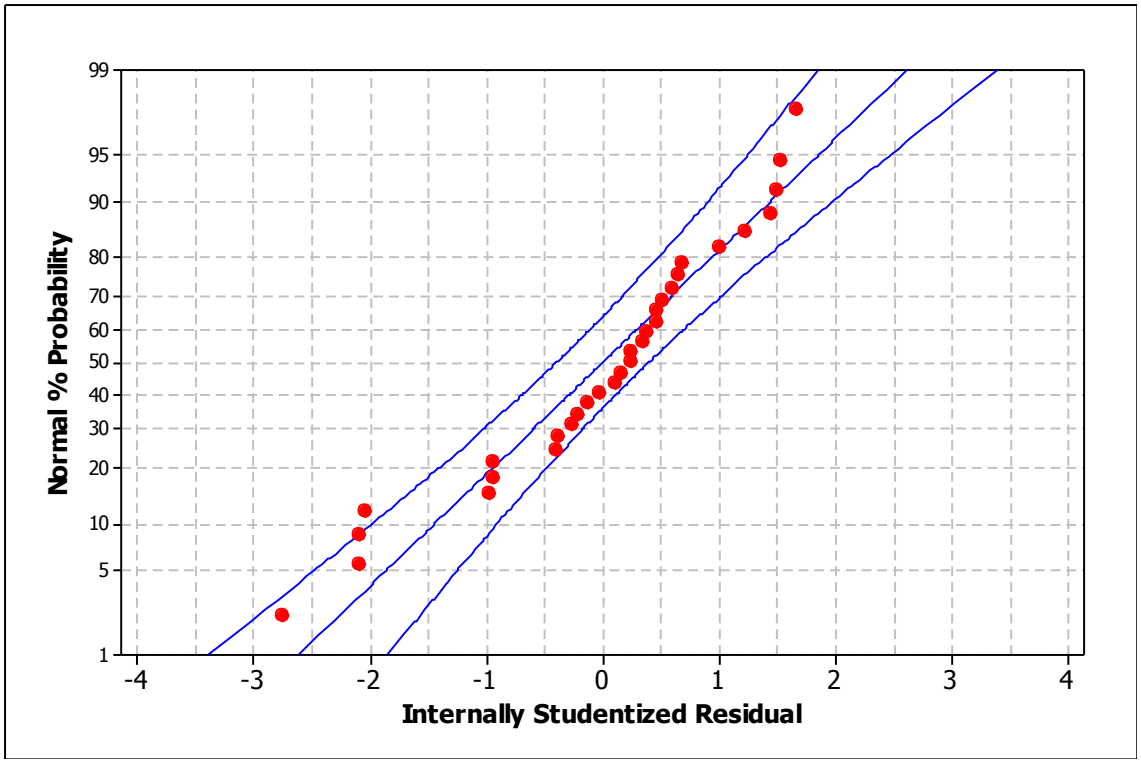


Figure 4.33: The normal probability of internally studentized residuals for % Biodegradation of 2,4-DCP.

4.3.3. Biodegradation kinetic of 2,4-DCP at RSM optimized condition

The biodegradation of 2,4-DCP by *Kocuria rhizophila* strain 11Y was carried out at RSM optimum conditions. The strain was able to degrade 2,4-DCP efficiently up to 400 mg/L at optimized conditions. The residual concentration of 2,4-DCP and growth profile has shown in Figure 4.34 and 4.35. The isolated strain was able to degrade up to 98% of 25 and 50 mg/L of 2,4-DCP whereas, around 70 to 85% degradation was observed for 75 to 350 mg/L of 2,4-DCP initial concentration. However, the degradation was drastically decreased to 41% for 400 mg/L of 2,4-DCP. In case of un-optimized conditions, the degradation observed for 300 mg/L of 2,4-DCP was 47% indicating that the degradation capacity of the isolate was greatly increased at optimized conditions. From the growth profile, it was observed that the inhibition effect was greatly prevailed after 200 mg/L of 2,4-DCP that was sharply increases for 400 mg/L. The lag phase was increased after 200 mg/L of 2,4-DCP showing inhibition effect. It was reported that at higher toxicity level, the microorganism use different bioenergetic strategies and gives more energy to biodegradation than biomass growth. While at lower toxicity, the microbes give more energy to biomass growth than biodegradation. So at higher 2,4-DCP concentration, a decreased proliferations of the microbes has been observed. However at 400 mg/L, the inhibition effect is large and the prolonged lag phase was observed which can be the reason for lower degradation at 400 mg/L. The dechlorination of 2,4-DCP has confirmed from the ESI mass spectroscopy and decrease in pH of the medium. Degradation of 2,4-DCP was confirmed from the HPLC (Figure 4.36) and ESI mass spectroscopy analysis. ESI mass spectroscopy shows the presence of single chlorinated and nonchlorinated compounds in the degradation products.

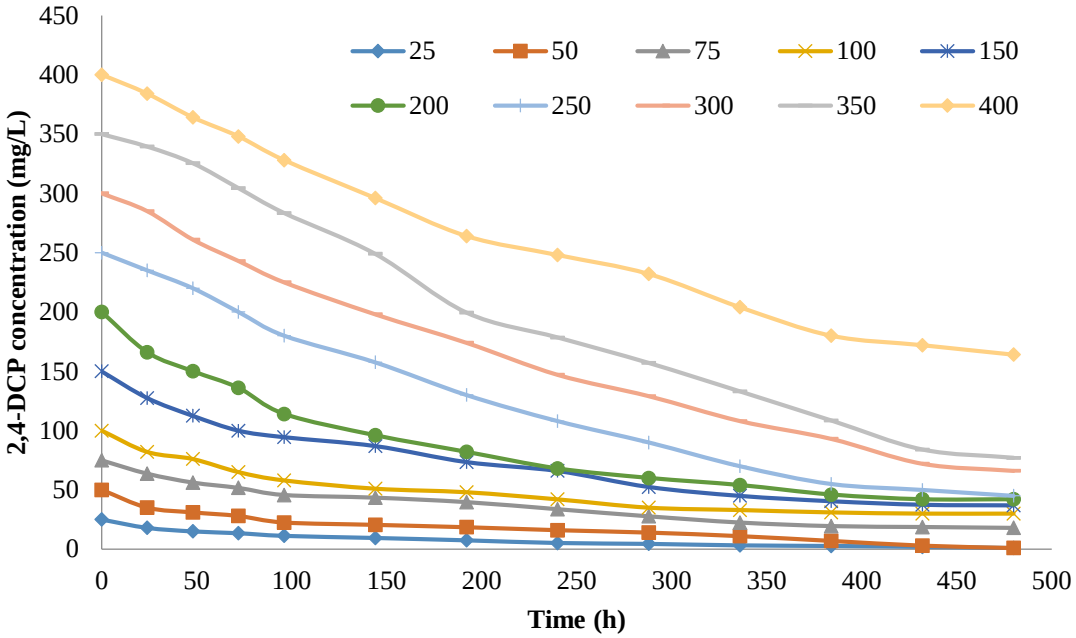


Figure 4.34: Residual concentration 2,4-DCP and its biodegradation profile by *K. Rhizophila* 11Y at a RSM optimized conditions.

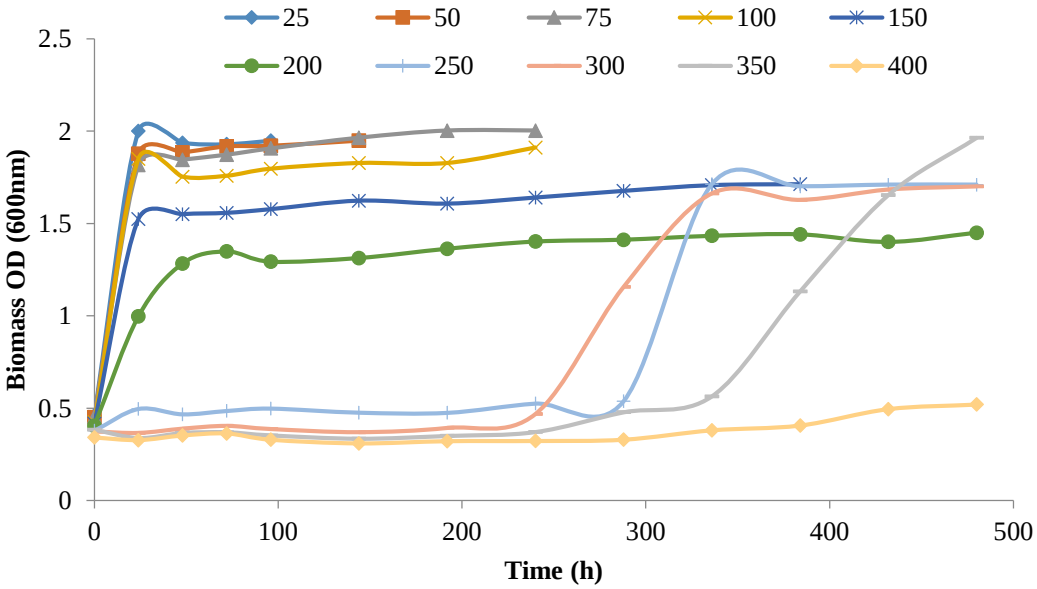


Figure 4.35: Biomass growth profile of *K. Rhizophila* 11Y at different initial 2,4-DCP concentration at a RSM optimized conditions.

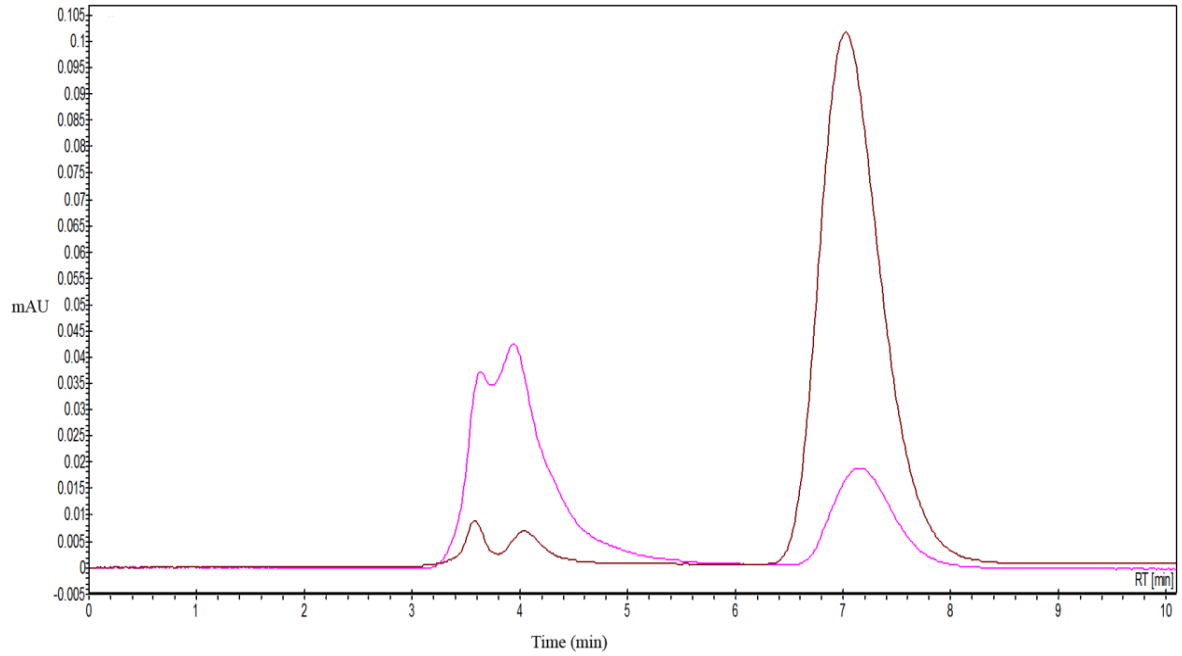


Figure 4.36: HPLC chromatogram showing the biodegradation of 300 mg/L of 2,4-DCP within 20 days. The retention time for 2,4-DCP is 7.12 min.

The plot between the *1/Rs* vs. *1/S* was plotted for experimental data (Figure 4.38). The plot is linear with the slope of *Ks/Rm* and the intercept of *1/Rm*. From the best fit line, the following values were obtained for biokinetic parameters using equation 3.1:

$\boldsymbol{R_m}$= 1.17 mg DCP/L/h and $\boldsymbol{K_s}$ = 568.1 mg/L

(R^2 = 0.986)

From figure 4.37, it can be observed that the substrate concentration where biodegradation rate is maximum is S_{max}= 350 mg/L. So, from Equation 3.5 the value of inhibition constant obtained was $\boldsymbol{K_i}$= 215.63 mg/L.

The biokinetic parameters obtained for biodegradation of 2,4-DCP by *Kocuria rhizophila* strain 11Y was in agreement with the literature. The maximum biodegradation rate (R_m) obtained in the present study was 1.17 mg DCP/L/h. Kargi and Eker (2005) [184] and Herrera et al. (2008) [11] reported the R_m value of 1.28 and 0.71 mg DCP/L/h for degradation of 2,4-DCP using *P. putida* CP1 and *Bacillus* consortium respectively. The half-saturation constant indicates the substrate affinity of the microorganism. Kargi and Eker (2005) [184] and Ma et al. (2012) [183] reported the K_s value of 427 and 175.2 mg/L for 2,4-DCP degradation by *P. putida* CP1 and aerobic granules respectively. A higher value of K_s (568.1 mg/L) obtained in the present study indicates good affinity of the isolate at low substrate concentration and microorganism attain it's the maximum removal rate at higher substrate concentration. The inhibition constant (K_i) is important as it expresses the inhibition effect of the substrate on the microorganism. A higher K_i indicates the less inhibition effect of substrate on the microorganism. The value of K_i obtained in the study was 215.63 mg/L that is in good correlation with the literature study [183-185]. Goswami et al. (2002) [179] and

Sahinkaya and Dilek (2007) [181] reported the K_i value of 44.46 and 81.34 mg/L respectively. The higher value of K_i indicates the higher resistant of *Kocuria rhizophila* 11Y for 2,4-DCP up to 400 mg/L showing the good potential of the isolate for removal of 2,4-DCP.

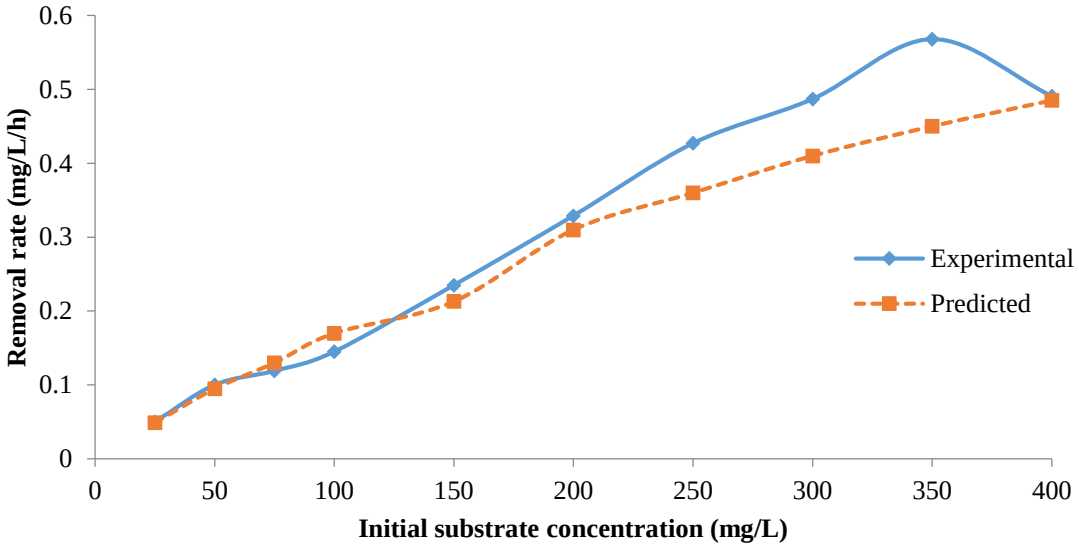


Figure 4.37: The effect of initial 2,4-DCP concentration on the removal rate by *K. rhizophila* 11Y.

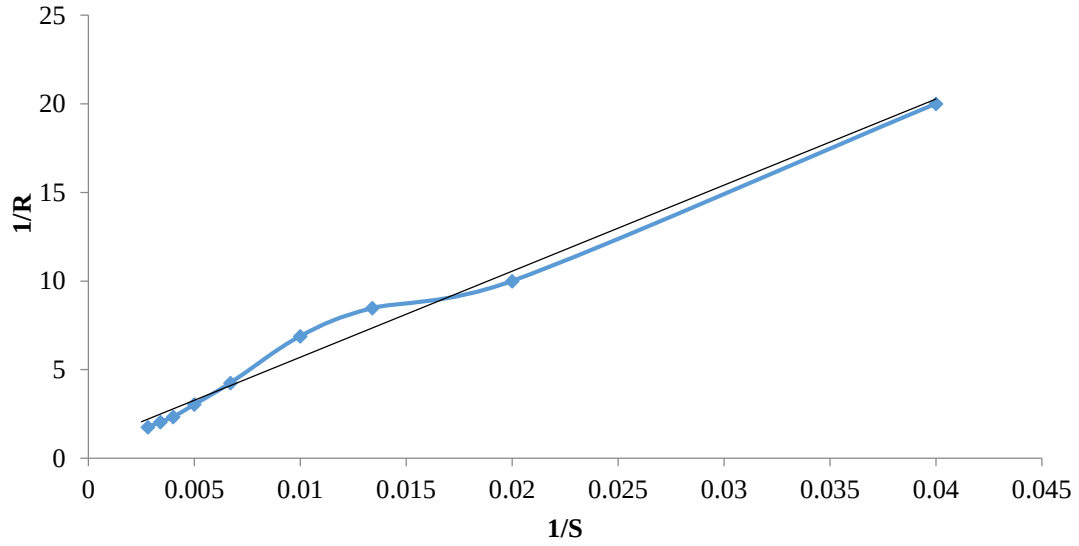


Figure 4.38: The double reciprocal plot of*1/R* v/s *1/S.*

4.3.4. Biodegradation of monochlorophenols

Biodegradation of three different monochlorophenols (2-CP, 3-CP, and 4-CP) by *Kocuria rhizophila* 11Y strain was carried out at RSM optimized conditions. Biodegradation of 2-CP for the initial substrate concentration of 25 to 400 mg/L was carried out by *Kocuria rhizophila* 11Y strain for 288 h. The abiotic loss observed for 2-CP was adjusted in the

presented result. The residual concentration of 2-CP with respect to time has shown in figure 3.39. The degradation rate was found to be increased with substrate concentration up to 400 mg/L of 2-CP. The strain has achieved 100% degradation of 25 mg/L initial 2-CP within 240 h. The strain attained 89, 88, 82, 71, and 61% degradation of 50, 100, 200, 300 and 400 mg/L of initial 2-CP respectively. The effect of initial substrate concentration on the removal rate was presented in figure 4.40. The removal rate increases almost linearly with substrate concentration up to 400 mg/L of initial 2-CP. The residual concentration increases with initial substrate concentration as shown in figure 4.41.

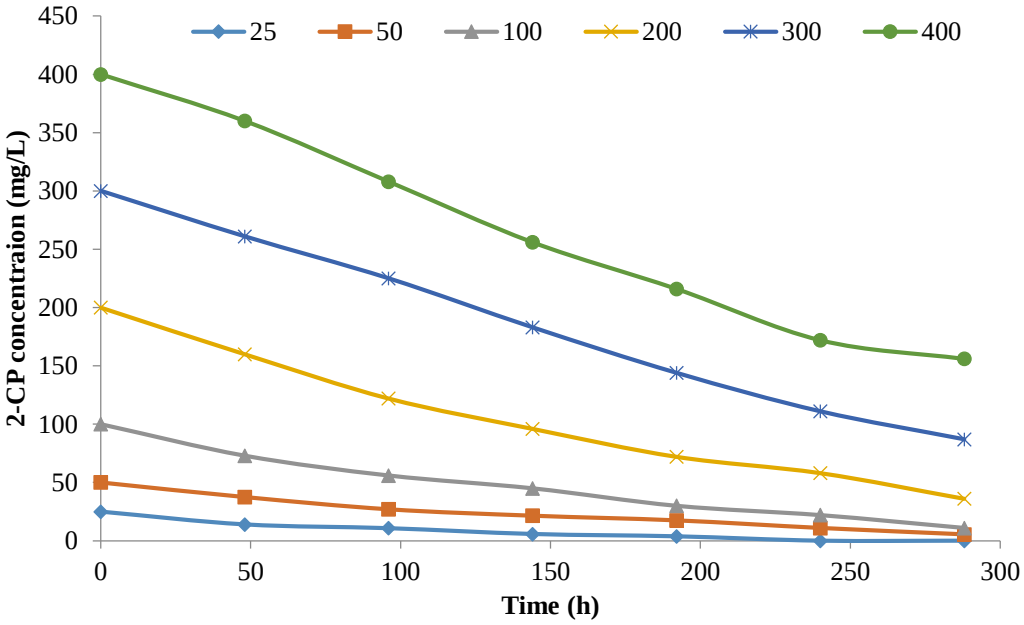


Figure 4.39: Biodegradation profile of 2-CP by *Kocuria rhizophila* 11Y at a different initial substrate concentration.

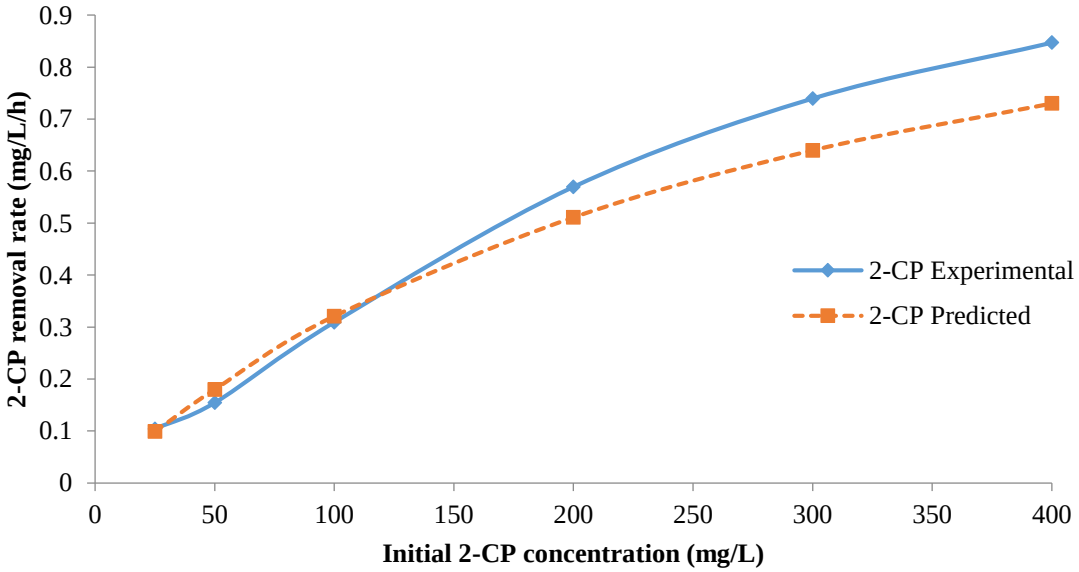


Figure 4.40: The effect of initial substrate concentration on removal rate of 2-CP by *Kocuria rhizophila* 11Y

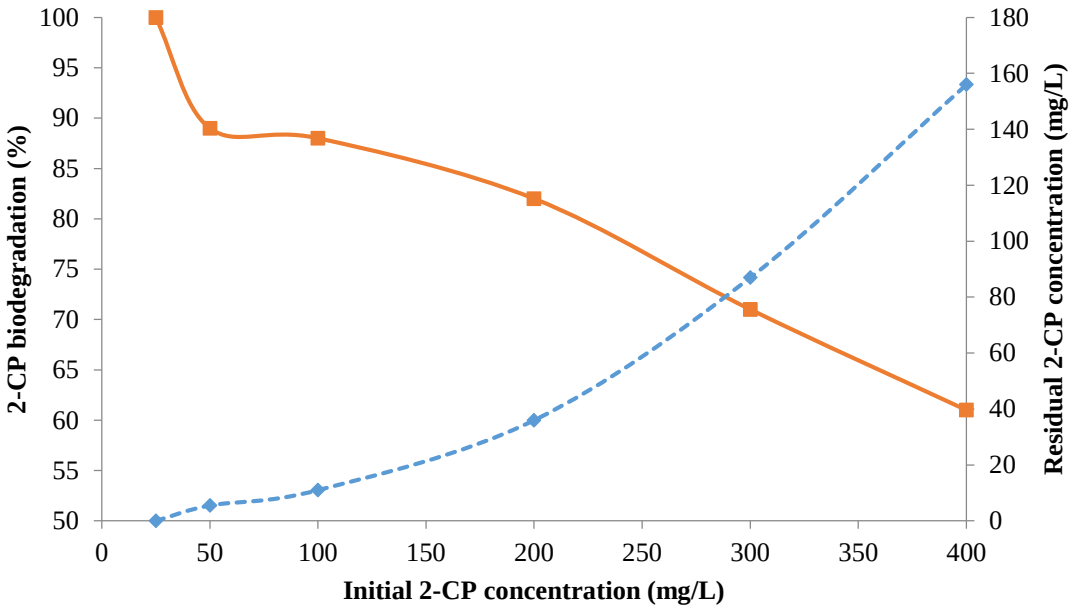


Figure 4.41: The effect of initial 2-CP concentration on percent biodegradation and residual concentration. Solid line indicates the 2-CP biodegradation and dotted line indicates the residual concentration.

In case of 3-CP and 4-CP, the degradation obtained was very low as compared 2-CP. The strain has shown low tolerant to 3-CP and 4-CP as evidenced by the very low growth rate. The biodegradation of 3-CP was performed for 336 h for the initial substrate concentration of 25 to 300 mg/L. The biodegradation profile of 3-CP at a different initial substrate concentration has shown in figure 4.42. The strain obtained 52, 34, 23, 11, 4, and 1% degradation for 25, 50, 75, 100, 200 and 300 mg/L of initial 3-CP concentration (Figure 4.44). The color of the medium turns to dark brown during the degradation process due to the accumulation of 3-chlorocatechol. This phenomenon was also reported in the literature. The brown color of the medium occurred when the microorganism degrades the chloroaromatic compounds via 3-chlorocatechol using *meta*- cleavage pathway. 3-chlorocatechol polymerizes due to auto oxidation which causes the brown color in the medium [40]. In case of 4-CP, the degradation was carried out for 192 h for the initial substrate concentration of 25 to 300 mg/L. The strain obtained 22, 19, 9, 5 and 1% degradation for initial substrate concentration of 25, 50, 100, 200 and 300 mg/L respectively (Figure 4.44). The biodegradation profile of 4-CP at a different initial substrate concentration was shown in figure 4.43. The effect of initial substrate concentration on the removal rate of 3-CP and 4-CP has shown in figure 4.45. The removal rate increases with substrate concentration and then its starts decreasing due to inhibition effect of 3-CP and 4-CP. Biodegradation of 3-CP via 3-chlorocatechol are results in dead-end pathway due to inhibition of enzyme catechol 2,3-dioxygenase. While in case of 4-CP, the production of intermediate compounds 5-chloro-2-hydroxymuconic semialdehyde, which is been also reported as dead-end metabolites [40].

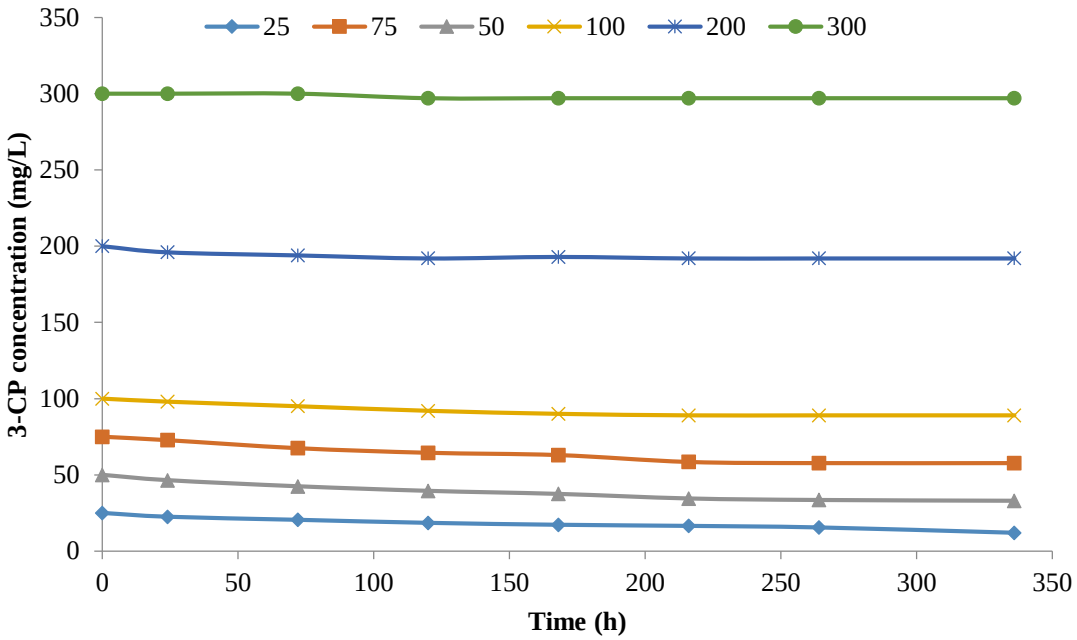


Figure 4.42: Biodegradation profile of 3-CP by *Kocuria rhizophila* 11Y at a different initial substrate concentration.

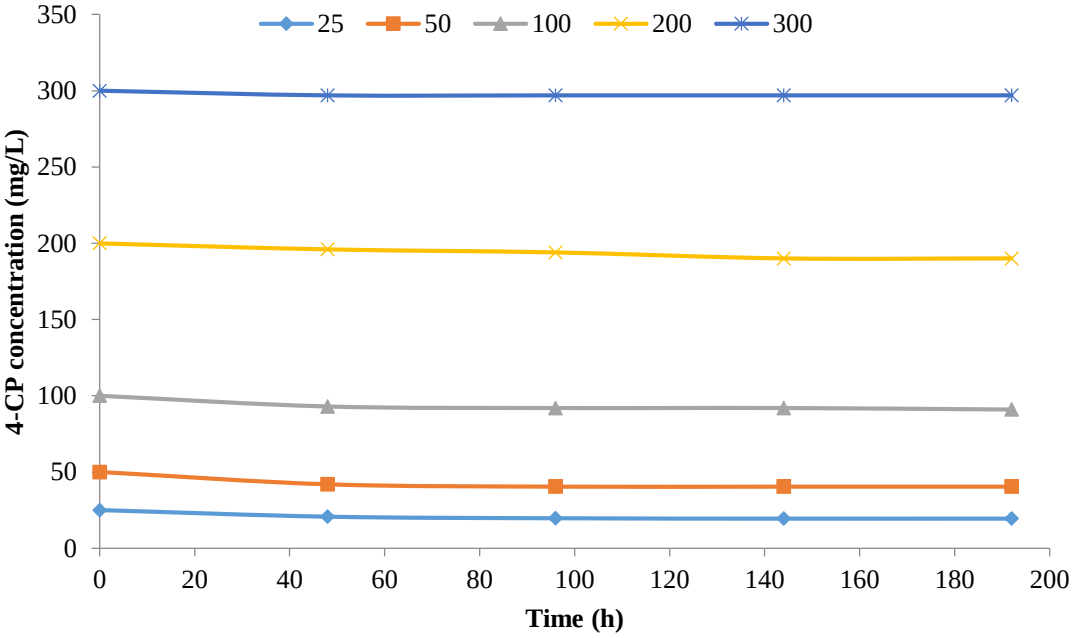


Figure 4.43: Biodegradation profile of 4-CP by *Kocuria rhizophila* 11Y at a different initial substrate concentration.

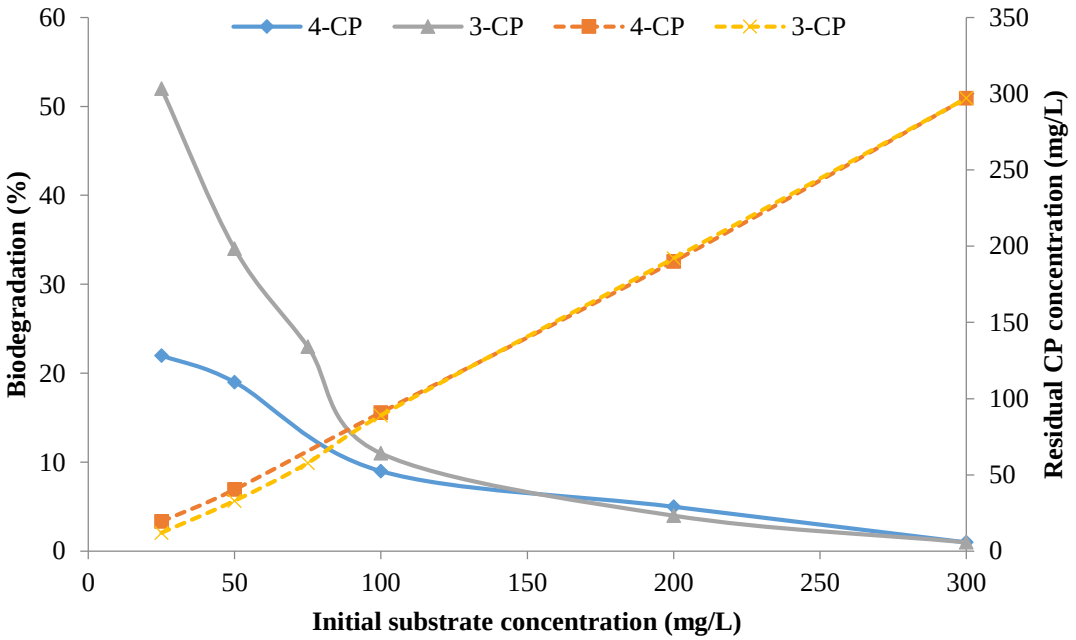


Figure 4.44: Biodegradation (%) and residual concentration of 3-CP and 4-CP at different initial substrate concentration. The solid line indicates the biodegradation (%) and the dotted line indicates the residual substrate concentration.

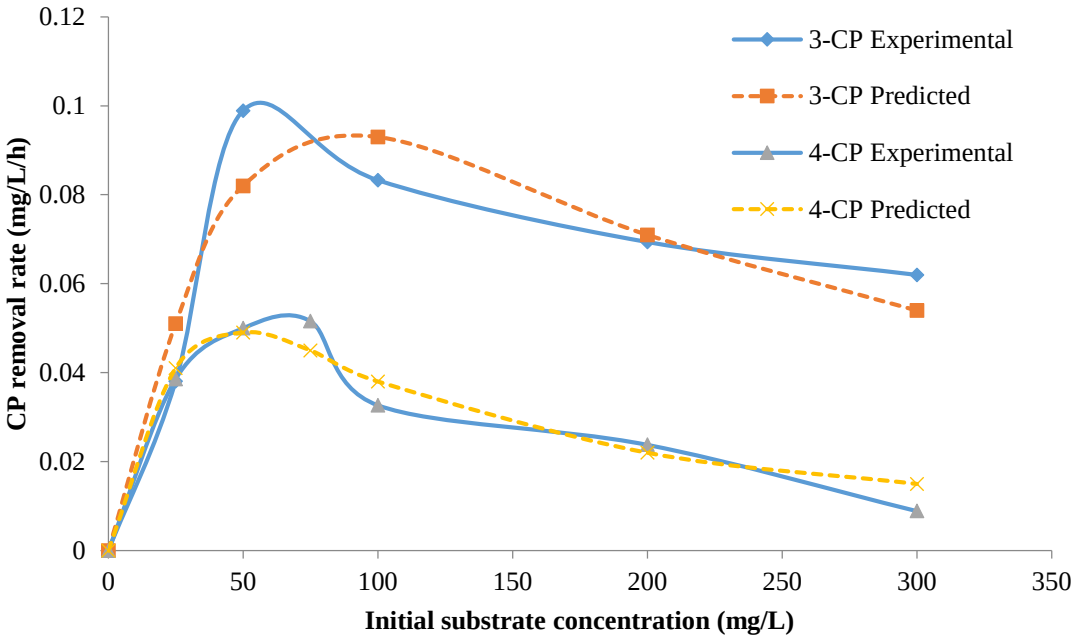


Figure 4.45: The effect of initial substrate concentration on removal rate of 3-CP and 4-CP by *Kocuria rhizophila* 11Y.

4.3.5. Biodegradation kinetic of monochlorophenols

Table 4.14 summarizes the biodegradation kinetic parameter for 2-CP, 3-CP and 4-CP using the Andrews substrate inhibition model (Equation 3.1). In case of 2-CP, the removal rate increases with substrate concentration up to 400 mg/L in this study (Figure 4.40). There was no inhibition of biodegradation process observed so the inhibition constant (K_i) can be neglected from the equation 3.1. So for 2-CP, the biodegradation kinetic parameters were obtained using equation 3.2 (Monod model). In case of 3-CP and 4-CP, the biodegradation kinetic follows the substrate inhibition model as the degradation rate decreases at higher concentration (Figure 4.45). Low inhibition constant for 3-CP and 4-CP indicates the higher substrate inhibition to the strain. The maximum removal rate obtained was 1.25, 0.384 and 0.8 mg/L/h for 2-CP, 3-CP and 4-CP respectively. This result was also in accordance that the toxicity of 2-CP is low, and that of 3-CP is higher among MCPs.

Table 4.14: Biodegradation kinetics parameters for monochlorophenols by *Kocuria rhizophila* 11Y using Andrews's model.

Compound	R_m **(mg/L/h)**	K_S **(mg/L)**	K_i **(mg/L)**	R^2
2-CP	1.25	289	-	0.97
3-CP	0.384	145.5	12.8	0.982
4-CP	0.8	336.5	23.8	0.894

4.3.6. Cometabolism of 2,4-DCP and monochlorophenols

The cometabolism of 2,4-DCP and monochlorophenols by *Kocuria rhizophila* 11Y was carried out in 50 mL MSM for 15 days. The combination of 2,4-DCP and MCPs used in the study was given in Table 4.15. Figure 4.46 and 4.47 shows the biodegradation obtained for 2,4-DCP and MCPs during the cometabolism.

Table 4.15: A different combination of 2,4-DCP and MCPs used for the co-metabolic study by *K. rhizophila* 11Y.

Compound	**DCP (mg/L)**	**2CP (mg/L)**	**3CP (mg/L)**	**4CP (mg/L)**	**Total CP (mg/L)**
DCP	100	-	-	-	100
DCP+2CP	100	50	-	-	150
DCP+3CP	100	-	50	-	150
DCP+4CP	100	-	-	50	150
DCP+2CP+3CP+4CP	100	25	12.5	12.5	150

The strain has achieved 65% biodegradation for 2,4-DCP alone for 100 mg/L of initial concentration. In the binary mixture of DCP and MCPs, the 2,4-DCP has shown 58, 64 and 65% biodegradation in the presence of 2-CP, 3CP and 4-CP respectively. The biodegradation of 2,4-DCP was decreased in the presence of 2-CP as compared to 2,4-DCP alone. The biodegradation of three MCPs in the binary mixture obtained was 68, 0 and 0% for 2-CP, 3-CP and 4-CP respectively. The strain was not able to utilize 3-CP and 4-CP in the presence of DCP. However, the strain was able to utilize DCP in the presence of 3-CP and 4-CP. The biodegradation (%) for the binary mixture was in the order of DCP>2CP>3CP≈4CP.

In the tertiary mixture, the biodegradation for 2,4-DCP obtained was 63% which was nearly equal to that obtained for 2,4-DCP alone. While for MCPs, only 2CP has shown 75% degradation in the tertiary mixture. There was no biodegradation obtained for 3-CP and 4-CP.

Overall the result shows that the strain has able to utilize the DCP in the presence of MCPs. However, the strain has shown no biodegradation of 3-CP and 4-CP during cometabolism. Among MCPs, the 2-CP has the lowest toxicity as compared to 3-CP and 4-CP. In the binary mixture, it was observed that the biodegradation of DCP was decreased in the presence of 2-CP. The reason may be that, the 2-CP was first utilized by the strain as it is less toxic than DCP and is readily degradable. The utilization of 2-CP leads to increase in biomass growth and because of that the strain has shown highest volumetric removal rate of total chlorophenols as shown figure 4.49. Although, the biodegradation of DCP is higher in the presence of 3-CP and 4-CP as compared to 2-CP, the total chlorophenol biodegradation (%) and total removal rate was much lower (Figure 4.48 and 4.49).The biodegradation of mixture of DCP and MCP was confirmed using HPLC. Figure 4.50 and 4.51 shows the HPLC chromatogram for biodegradation of mixture of 2-CP+ DCP and 3-CP+DCP at 0 and 144 h. There was no degradation of 3-CP observed, but the strain has shown degradation of DCP and 2-CP.

Even though the toxicity of DCP is higher than 3-CP and 4-CP, the strain was only able to utilize DCP but not able to degraded 3-CP and 4-CP. Molecular structure and chlorine substitution on benzene ring have an effect on biodegradation of chlorophenols. The removal of chloride at *meta* and *para* position is more energy demanding process due to electron hindrance to the enzyme. Single bacteria may not possess all the enzymes requires for degradation of different chlorophenol congener. Also, the acclimatization process has a great influence on the genetic structure or enzyme expression in the microorganism. This observation was also reported in the literature. Boyd and Shelton (1984) studied the effect of acclimatization of sludge to specific chlorophenol congener and cross-acclimation on the degradation of monochlorophenols and dichlorophenols. They observed the existence of two unique microbial activities that were in turn different from fresh sludge [229]. In the presence study, the isolate was acclimated to 2,4-DCP and hence express the enzymes that are only responsible for utilization of 2,4-DCP and 2-CP. 2-CP is structurally more similar to 2,4-DCP and also the degradation product of 2,4-DCP.

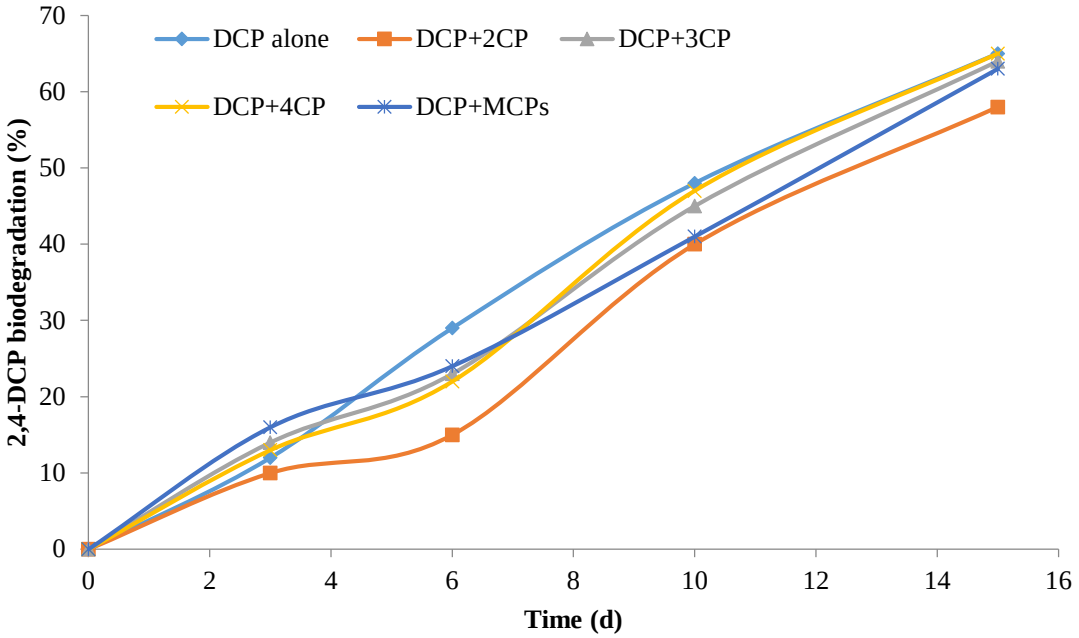


Figure 4.46: Biodegradation of 2,4-DCP by *Kocuria rhizophila* 11Y in the presence of monochlorophenols.

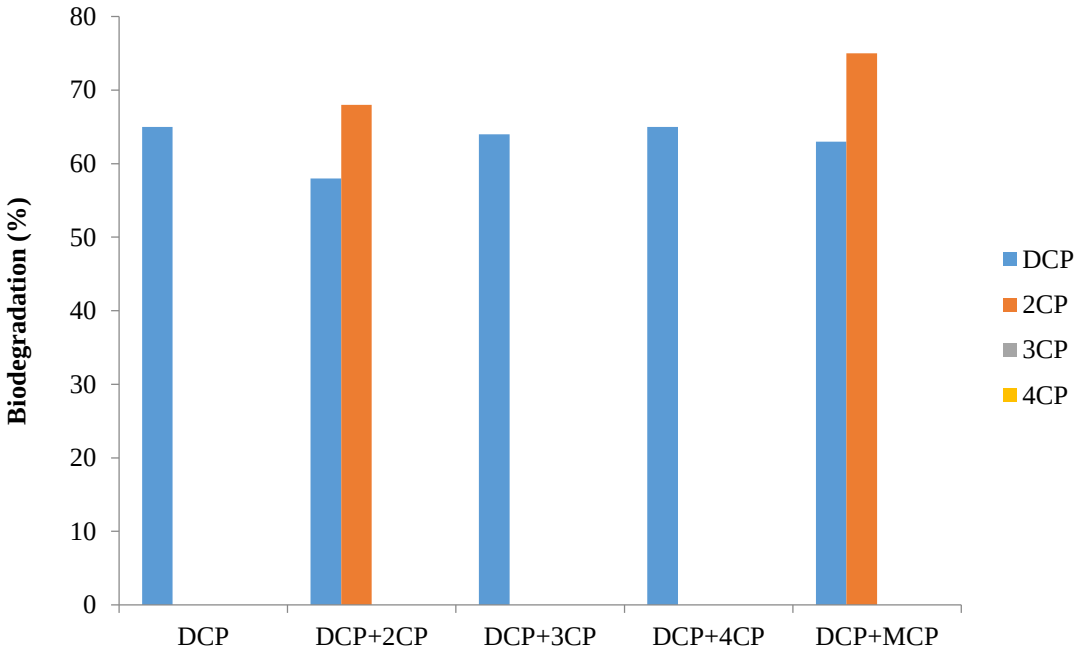


Figure 4.47: Biodegradation (%) of different chlorophenols during the cometabolic study by *Kocuria rhizophila*11Y.

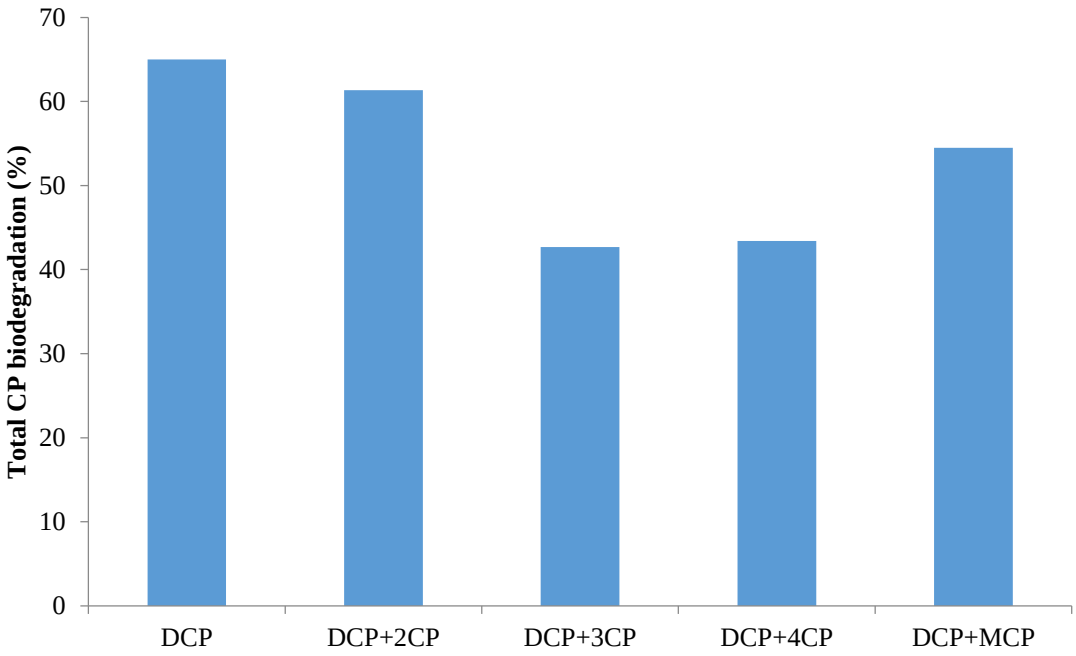


Figure 4.48: Total chlorophenol biodegradation for a different combination of chlorophenols by *Kocuria rhizophila* 11Y.

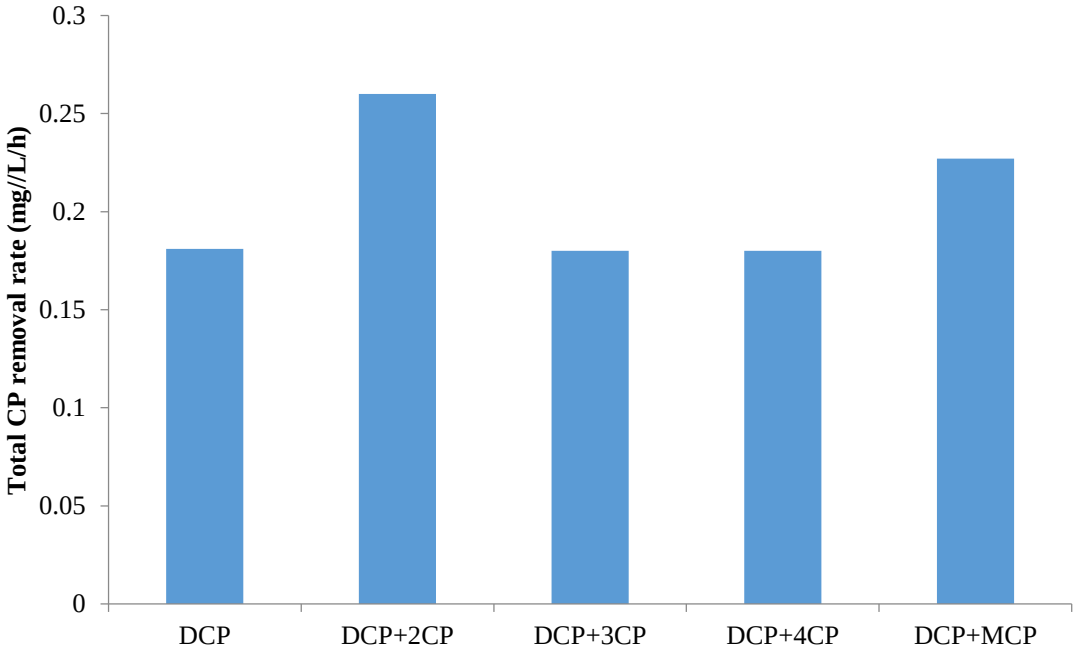


Figure 4.49: Total chlorophenol removal rate for a different combination of chlorophenols by *Kocuria rhizophila* 11Y.

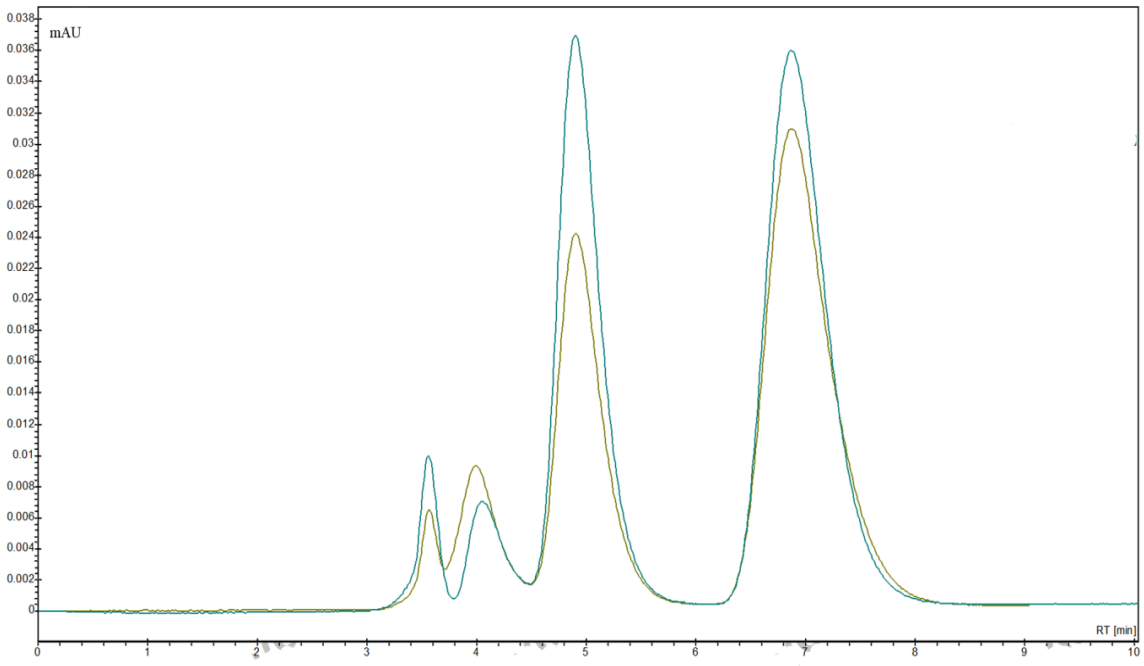


Figure 4.50: HPLC chromatogram of cometabolic degradation of 2-CP (50 mg/L) and 2,4-DCP (100 mg/L) by *Kocuria rhizophila* 11Y between 0 h and 144 h. The retention time for 2-CP and 2,4-DCP is 4.91 and 6.88 min respectively.

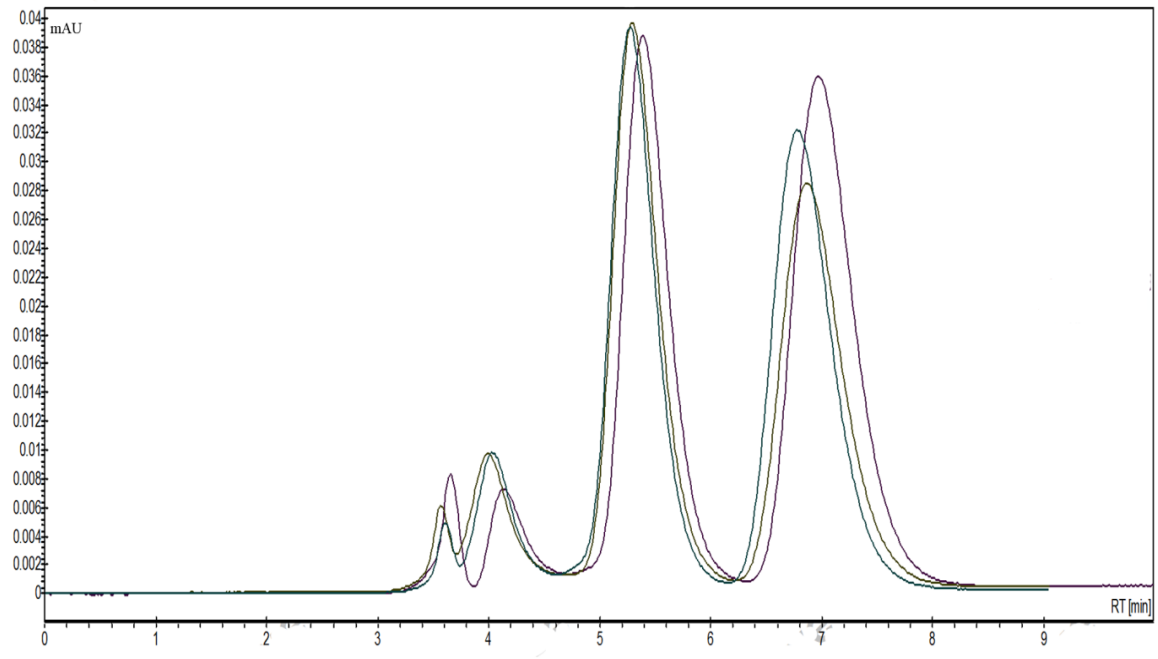


Figure 4.51: HPLC chromatogram of cometabolic degradation of 3-CP (50 mg/L) and 2,4-DCP (100 mg/L) by *Kocuria rhizophila* 11Y at 0, 72 and 144 h. The retention time for 3-CP and 2,4-DCP is 5.37 and 6.9 min respectively.

4.4. Biodegradation of chlorophenols by *Pseudomonas aeruginosa* strain GF: Kinetic and cometabolism

In the present section, the biodegradation and its kinetic of six different chlorophenols including 2-CP, 3-CP, 4-CP, 2,4-DCP, 2,4,6-TCP and PCP by ***Pseudomonas aeruginosa* strain GF** were studied. The cometabolism of 2,4-DCP in the presence of all the three monochlorophenols by the isolate were evaluated. The isolate was not able to degrade 2,4,6-TCP and PCP while it has shown only negligible degradation for 3-CP and 4-CP. However, the strain has shown great efficiency for removal of 2-CP and 2,4-DCP. The ***Pseudomonas aeruginosa* strain GF** was able to tolerate high concentration of 2,4-DCP up to 700 mg/L,which was higher than that observed in previous two isolated strains.

The optimization of the experimental parameters shows that the strain shows the similar characteristic of both previous strains as they were isolated from the same source (data not shown). So, no detailed optimization study was performed and the RSM optimized condition obtained for *B. endophyticus* CP1R was used for the isolate *P. aeruginosa* strain GF. So all the biodegradation experiments were conducted using the following condition: pH 7.36, temperature 35.1°C, inoculum size 10 %(v/v) and $(NH_4)_2SO_4$ concentration 1.4 g/L.

4.4.1. Biodegradation of 2,4-DCP

Biodegradation of 2,4-DCP by *P. aeruginosa* strain GF was carried out at a different initial concentration ranging from 50 to 700 mg/L for 15 days. The effect of initial substrate concentration on the biodegradation of 2,4-DCP has been shown in figure 4.52. The isolated strain was not able to degrade 2,4-DCP completely. Initially, the rate of degradation was observed to be high and later on it started to slow down gradually. The biodegradation rate increased with initial substrate concentration up to 500 mg/L, and then it started to decrease. The strain showed 26 to 68% degradation of 2,4-DCP for the initial substrate concentration of 50 to 700 mg/L. The percent biodegradation and residual concentration at different initial substrate concentration havebeen depicted in figure 4.53. The percent biodegradation decreases and the residual concentration increases with increasing substrate concentration. Figure 4.54 shows the effect of initial substrate concentration on the removal rate of 2,4-DCP. The removal rate was increased linearly up to 500 mg/L and remains almost constant up to 600 mg/L. The removal rate was observed to decrease with 700 mg/L. The reduction in degradation rate observed may be due to inhibition imposed by the accumulation of metabolites. Another factor that affects the degradation rate is the cell death occurring due to the toxicity of chlorophenol at a higher concentration.

Biodegradation of MCPs and 2,4-DCP has been confirmed by the HPLC and mass spectroscopy analysis (Figure 4.55 and 4.56). Different metabolites formation was observed in ethyl acetate extract of biodegradation sample of 2,4-DCP (50 mg/L) by ESI-MS data (figure 4.55). Different m/z peaks were identified in the spectra that represent the biodegradation products. The highest peak (m/z=370) might related to the conjugation product of two sodium 2,4-dichlorophenoxy ion [225]. The m/z peak 201, 199, 198, 141, and 79 might be corresponding to 3,5-dichlorocatechol [218]. The multiplets (170, 152, 129, 111,

and 101) were identified as monochlorophenol and its conjugate fragments. Other m/z peaks (354, 242, 223, and 180) related to the degradation product or conjugate fragment were also detected.

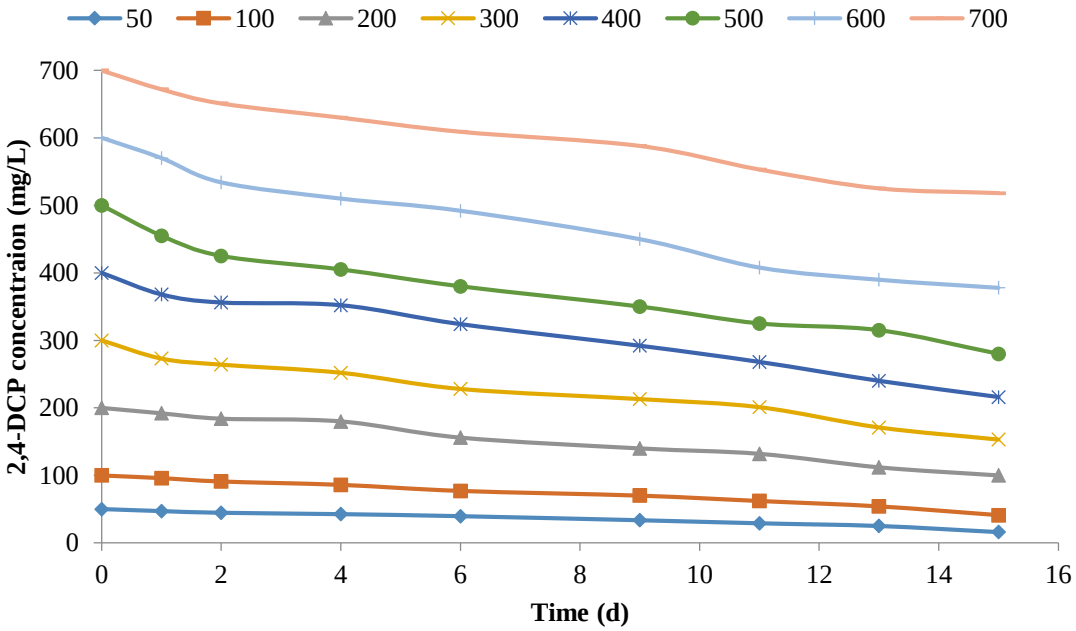


Figure 4.52: Biodegradation profile of 2,4-DCP with time at a different initial concentration by *P. aeruginosa* GF.

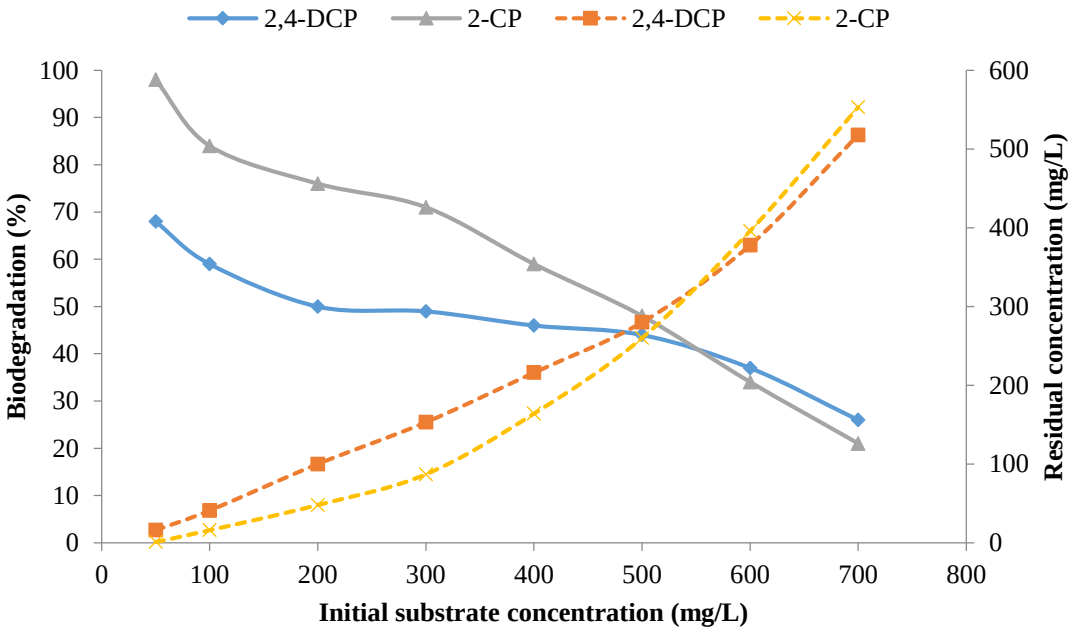


Figure 4.53: Effect of initial substrate concentration on biodegradation and residual concentration for 2-CP and 2,4-DCP. The solid line indicates the biodegradation (%) and the dotted line indicates the residual substrate concentration.

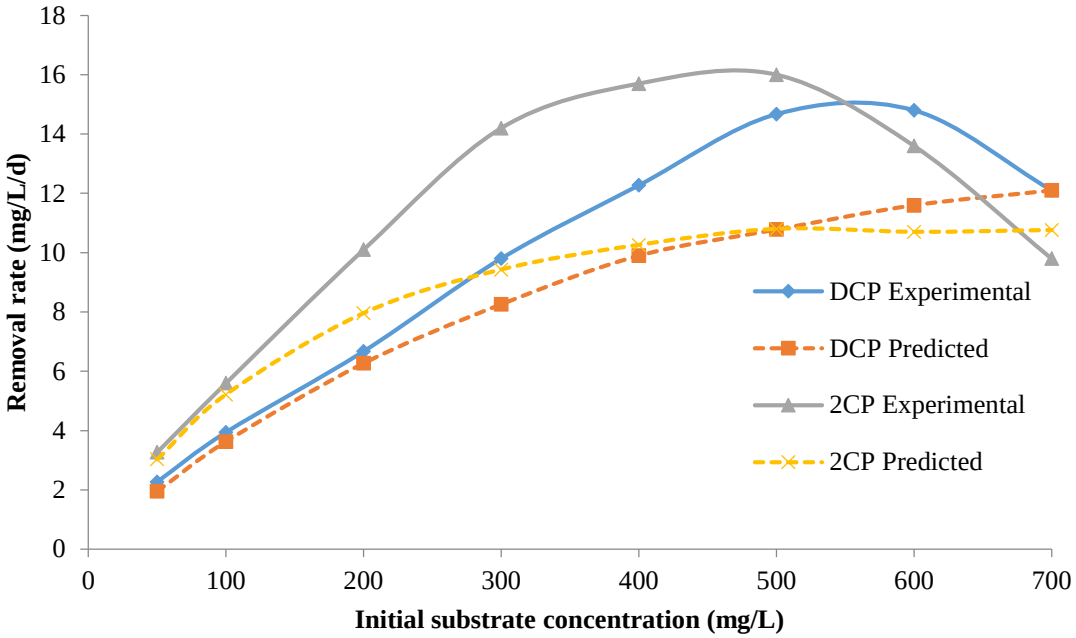


Figure 4.54: Effect of initial substrate concentration on the removal rate of 2-CP and 2,4-DCP by *P. aeruginosa* GF.

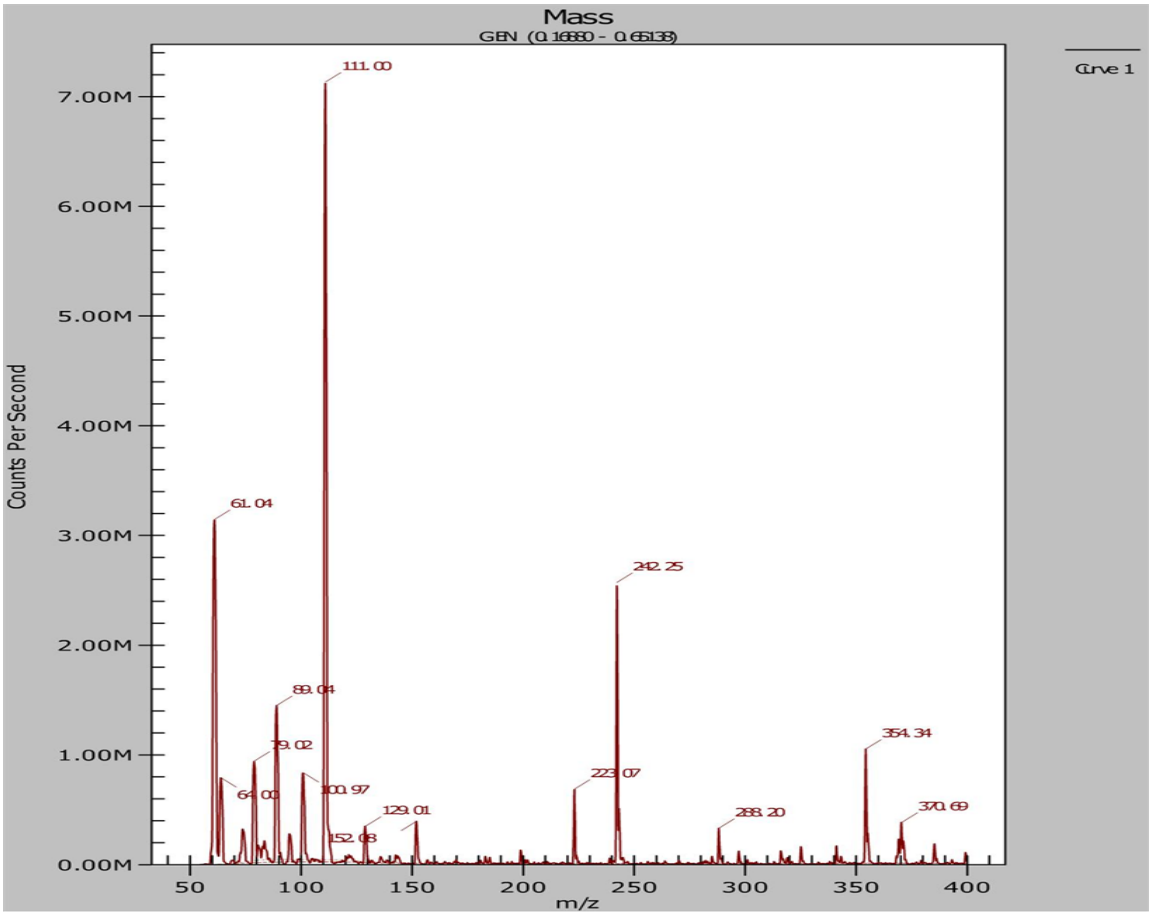


Figure 4.55: Mass spectroscopy of the biodegradation product of 2,4-DCP (50 mg/L) after 15 days.

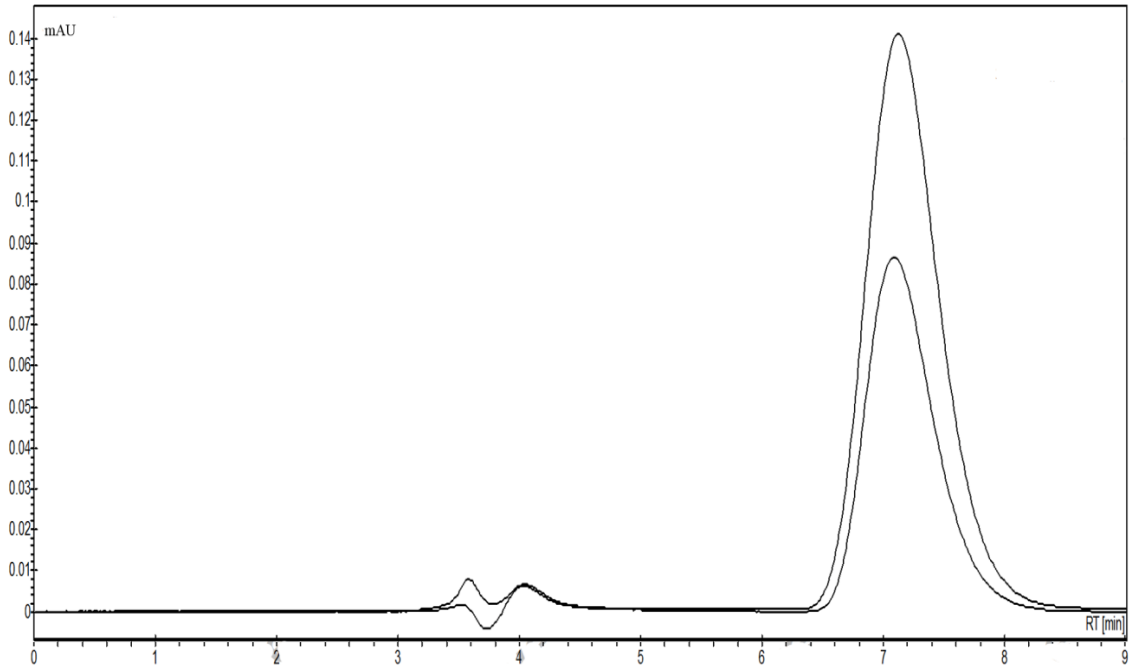


Figure 4.56: HPLC chromatogram of 2,4-DCP (400 mg/L) degradation by *P. aeruginosa* GF at 0 h and 360 h.

4.4.2. Biodegradation of Monochlorophenols

The biodegradation of 2-CP, 3-CP and 4-CP was carried out by *P. aeruginosa* strain GF at different initial concentration for 15 days. In case of 2-CP, the study has been performed for the initial substrate concentration of 50 to 700 mg/L. The effect of initial substrate concentration on the biodegradation of 2-CP has shown in figure 4.57. The strain has achieved up to 98, 84, 76 and 71% degradation for an initial concentration of 50, 100, 200 and 300 mg/L respectively. After 300 mg/L of initial substrate concentration, the biodegradation rate decreases. The biodegradation rate obtained for the initial substrate concentration of 400, 500, 600, and 700 mg/L were 59, 48, 34 and 21% respectively. The change observed in percent biodegradation, and residual concentration with initial substrate concentration for 2-CP has been presented in figure 4.53. The percent biodegradation decreases with increasing substrate concentration, and it decreases at a higher rate after 300 mg/L of initial substrate concentration as shown in figure 4.53.

The effect of initial substrate concentration on the removal rate of 2-CP has been depicted in figure 4.54. The removal rate increases with substrate concentration up to 400 mg/L and remains almost constant till 500 mg/L of initial concentration. After 500 mg/L, the removal rate decreases up to 700 mg/L. The removal rate observed for 2-CP tends to be higher than that of 2,4-DCP at a lower concentration. However after 500 mg/L of initial substrate concentration, the removal rate found to be lower for 2-CP than that of 2,4-DCP.

The biodegradation of 3-CP and 4-CP by *P. aeruginosa* strain GF were performed at 50 and 100 mg/L concentration for 15 days. It has been observed that 3-CP and 4-CP confer higher inhibition effect on the strain. The strain was unable to grow and tolerate at higher

concentration of both the compounds. In case of 3-CP, the strain has shown 29 and 14% degradation for 50 and 100 mg/L of initial concentration. While in case of 4-CP, the strain has shown 42 and 27% degradation for 50 and 100 mg/L of initial concentration respectively. The residual concentration of both the compounds has been shown in figure 4.58. Figure 4.59 shows the HPLC chromatogram for biodegradation of 2-CP (300 mg/L) at o and 360 h which confirms the degradation of 2-CP. There were no intermediate products were detected during HPLC analysis.

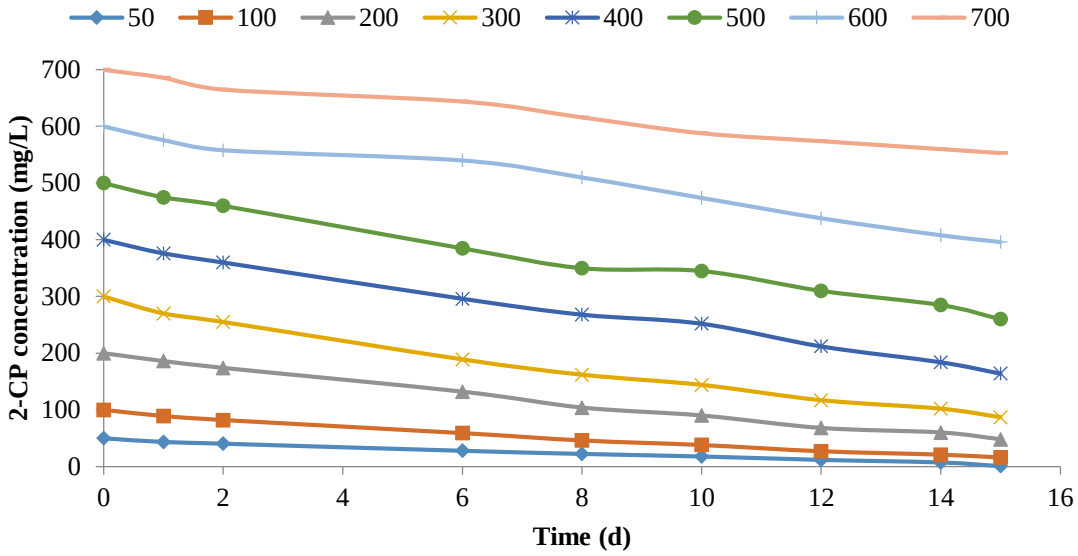


Figure 4.57: Biodegradation profile of 2-CP with time at a different initial concentration by *P. aeruginosa* GF.

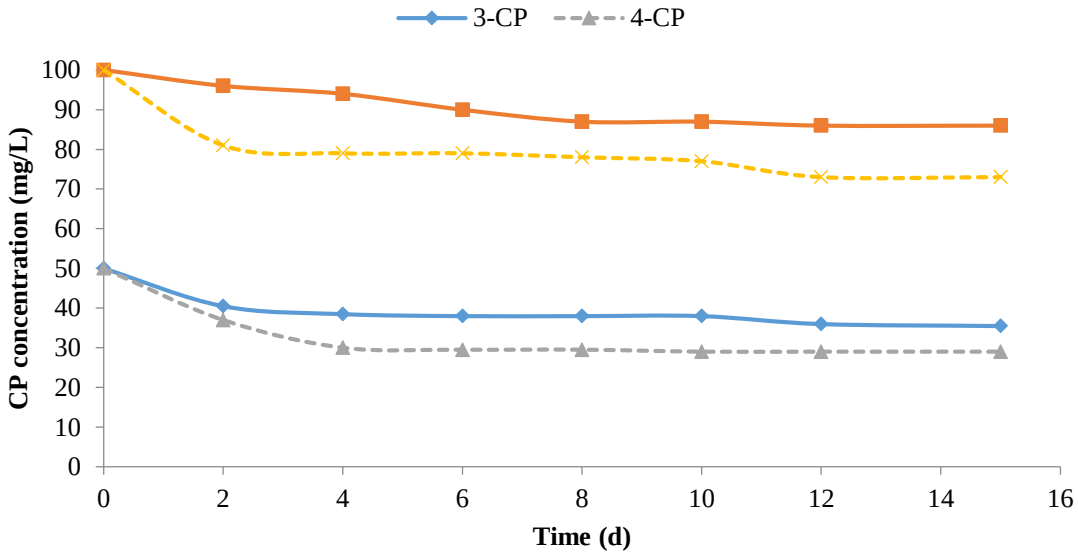


Figure 4.58: Biodegradation profile of 3-CP and 4-CP with time at a different initial concentration by *P. aeruginosa* GF.

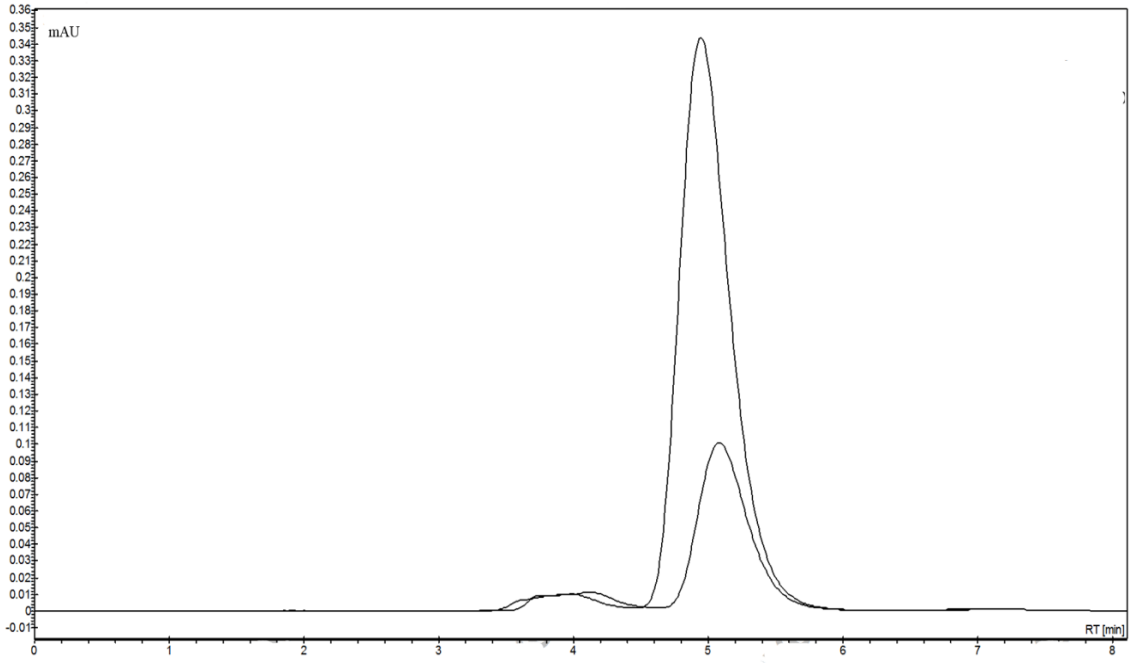


Figure 4.59: HPLC chromatogram of 2-CP (300 mg/L) degradation by *P. aeruginosa* GF at 0 h and 360 h.

4.4.3. Biodegradation kinetic of MCPs and 2,4-DCP

Table 4.16 summarizes the biokinetic parameters for 2-CP, 3-CP, 4-CP and 2,4-DCP degradation obtained using the Andrews substrate inhibition model as mentioned above (Equation 3.1). The biokinetic parameters for 2-CP and 2,4-DCP were calculated using the Matlab 6 and Lineweaver-Burk plot. The maximum removal rate obtained for 2-CP and 2,4-DCP were 1.56 and 2.03 mg/L/h respectively. The inhibition constant (K_i) been an important bio-kinetic parameter that determines the inhibition effect of the toxic compound on the microorganism. Higher the inhibition constant; lower is the inhibition effect on the microorganism. The inhibition constant (K_i) obtained for 2-CP and 2,4-DCP were 667.55 mg/L and 1340.8 mg/L respectively. The lower inhibition constant for 2-CP shows that it has higher inhibition effect on the microbes than 2,4-DCP. Also, the percent biodegradation and removal rate was also observed to be high in case of 2-CP as compare to 2,4-DCP (Figure 4.53 and 4.54). The value of half saturation constant obtained for 2-CP and 2,4-DCP were 524.34 and 1145 mg/L respectively. The lower value of half-saturation constant (*Ks*) for 2-CP shows that it has achieved the maximum removal rate at a lower concentration as compared to 2,4-DCP. The critical substrate concentration (S_{max} *or* S^*), as per equation 3.5, were determined as 591.6 and 1239 mg/L for 2-CP and 2,4-DCP respectively.

Table 4.16: Biodegradation kinetic constants obtained for 2,4-DCP and MCPs using Andrew's model for *P. aeruginosa* GF.

Compound	R_m **(mg/L/h)**	K_S **(mg/L)**	K_i **(mg/L)**	R^2
2,4-DCP	2.03	1145	1340.8	96.5
2-CP	1.56	524.34	667.55	88.6
3-CP	0.06	15.4	244.5	99.9
4-CP	0.117	48.47	1251	99.8

The biodegradation kinetic parameters for 3-CP and 4-CP were calculated using the Graph Pad Prism-5. The maximum removal rate obtained for 3-CP and 4-CP were 0.06 and 0.117 mg/L/h respectively. The half-saturation constant (*Ks*) obtained were 15.4 and 48.47 mg/L for 3-CP and 4-CP respectively. The low value of half saturation constant suggests the isolated strain has low affinity for 3-CP and 4-CP. The inhibition constant for 3-CP and 4-CP obtained was 244.5 and 1251 mg/L.

4.4.4. Cometabolic study of MCPs and 2,4-DCP

The cometabolic study was carried out to analyze biodegradation of 2,4-DCP in the presence of MCPs (2-CP, 3-CP, and 4-CP) by the isolate. The cometabolic study was performed in 250 mL Erlenmeyer with 50 mL MSM. A different combination of 2,4-DCP and MCPs has been added to the flask as mentioned in Table 4.17. Samples were withdrawn at a regular interval for HPLC analysis of residual chlorophenols concentration. The cometabolic study of 2,4-DCP (DCP for further discussion) in the presence of three different MCPs by *P. aeruginosa* strain GF was analyzed and the result has been shown in figure 4.60.

Table 4.17: A different combination of 2,4-DCP and MCPs used for the co-metabolic study by *P. aeruginosa* GF.

Compound	**DCP (mg/L)**	**2CP (mg/L)**	**3CP (mg/L)**	**4CP (mg/L)**	**Total CP (mg/L)**
DCP	100	-	-	-	100
DCP+2CP	100	50	-	-	150
DCP+3CP	100	-	50	-	150
DCP+4CP	100	-	-	50	150
DCP+2CP+3CP+4CP	100	25	12.5	12.5	150

The isolate has shown 60% biodegradation of DCP alone at 100 mg/L of initial substrate concentration. In the binary mixture of MCPs and DCP, the biodegradation observed for DCP were 46, 46 and 51% in the presence of 2-CP, 3-CP and 4-CP respectively. The degradation of DCP in the presence of MCPs obtained was lower than the biodegradation of DCP alone. While the degradation of 2-CP, 3-CP, and 4-CP obtained in the binary mixture were 41, 1 and 1% respectively. The total chlorophenol removal obtained was 44.34, 31 and 34.4% in the presence of 2-CP, 3-CP and 4-CP respectively which was lower than that obtained for DCP i.e. 60%.

In the quaternary mixture of MCPs and DCP, the biodegradation obtained for DCP was 38% of initial substrate concentration. The degradation of DCP obtained in the quaternary mixture has been lowest compared to DCP alone and the binary mixtures. The degradation observed for 2-CP, 3-CP and 4-CP were 35, 0 and 2% in the mixture respectively. The total chlorophenol removal in the quaternary mixture was found to be 37.4%. The biodegradation of DCP and MCPs by the *P. aeruginosa* GF takes place in the order of DCP>2CP>4CP>3CP in the quaternary mixture. Total chlorophenol degradation (%) was observed in the order of DCP> (DCP+2CP)> (DCP+MCPs)> (DCP+4CP)> (DCP+3CP).

Figure 4.61 depicts the total chlorophenol removal rate obtained for the different combination of DCP and MCPs. The highest total chlorophenol removal observed was 0.184 mg/L/h for a combination of 2-CP and DCP. The total chlorophenol removal rate observed was in the order of (DCP+2CP)> DCP> (DCP+MCPs)> (DCP+4CP)> (DCP+3CP). The toxicity of MCPs and DCP was in the order of 2CP<4CP<3CP<DCP. DCP tends to be more toxic than MCPs but in this study, DCP has shown more degradation than that of MCPs. In the binary and quaternary mixture, the degradation of DCP was found to be higher than that of MCPs. The degradation of 3-CP and 4-CP in the presence of DCP has been inhibited completely as compared to 2-CP.

Similar results have also been reported, in which higher toxic compounds were observed to be degraded faster compared to the relatively lower toxic compounds. Zilouei et al. (2006) [24] reported the biodegradation of 2,4-DCP and 2,4,6-TCP to be higher as compared to 4-CP and 2-CP by the mixed consortium. The order of removal increases as TCP>DCP>4CP>2CP. The easily degradable 2-CP was removed at the slowest rate. However, the exact mechanism for this phenomenon has not been reported by the authors. Another study also reported similar findings during the cometabolism of MCPs by *P. aeruginosa* strain. The *Pseudomonas* strain was able to utilize 3-CP and 4-CP, both singly and in the mixture up to 25 mg/L while the strain was unable to utilize 2-CP in the mixture [223]. Farrell and Quilty (1999) [40] have also reported the degradation of MCPs by the mixed consortium in which the degradation of 4-CP and 3-CP were reported to be higher as compared to 2-CP. Papazi and Kotzabasis (2013) have reported that the microorganisms utilize different bio-energetic processes for degradation of compounds depending on their toxicity. In the presence of higher toxic compounds or when toxicity of compounds reaches a threshold level, the microorganism gives more energy to toxicity removal or biodegradation than to biomass growth. They have also reported that the biodegradation of DCP congeners 2,3-DCP, 2,5-DCP and 3,4-DCP (higher toxicity, one *meta*substitution) was higher compared

to DCP congeners 2,4-DCP and 2,6-DCP (lower toxicity, no *meta* substitution) and the corresponding MCPs [224].

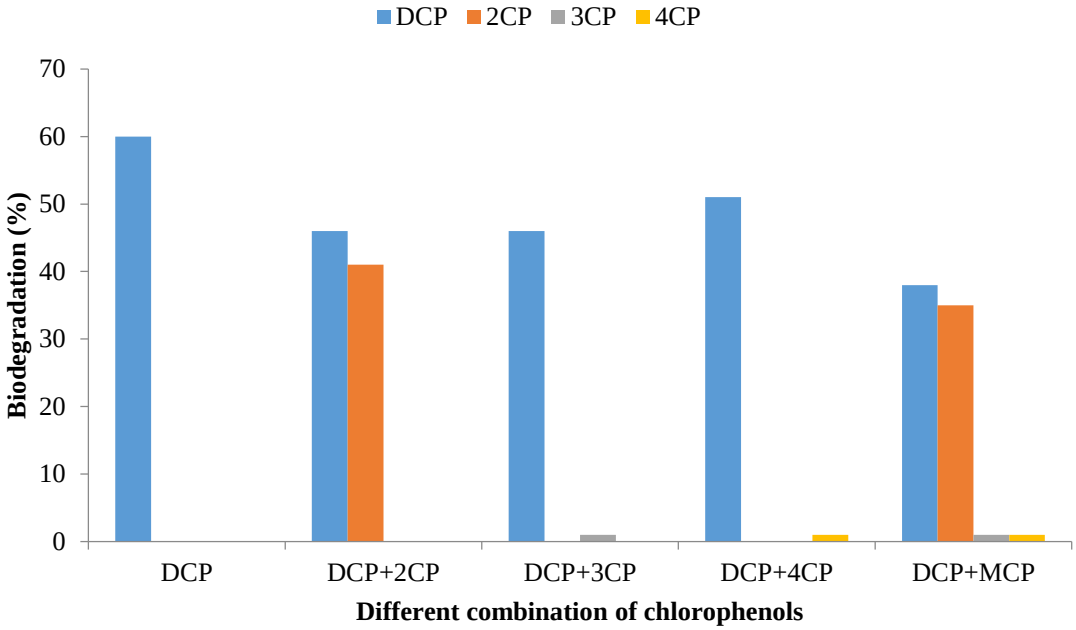


Figure 4.60: Biodegradation (%) observed for 2,4-DCP and MCPs during the cometabolic study by *P. aeruginosa* GF.

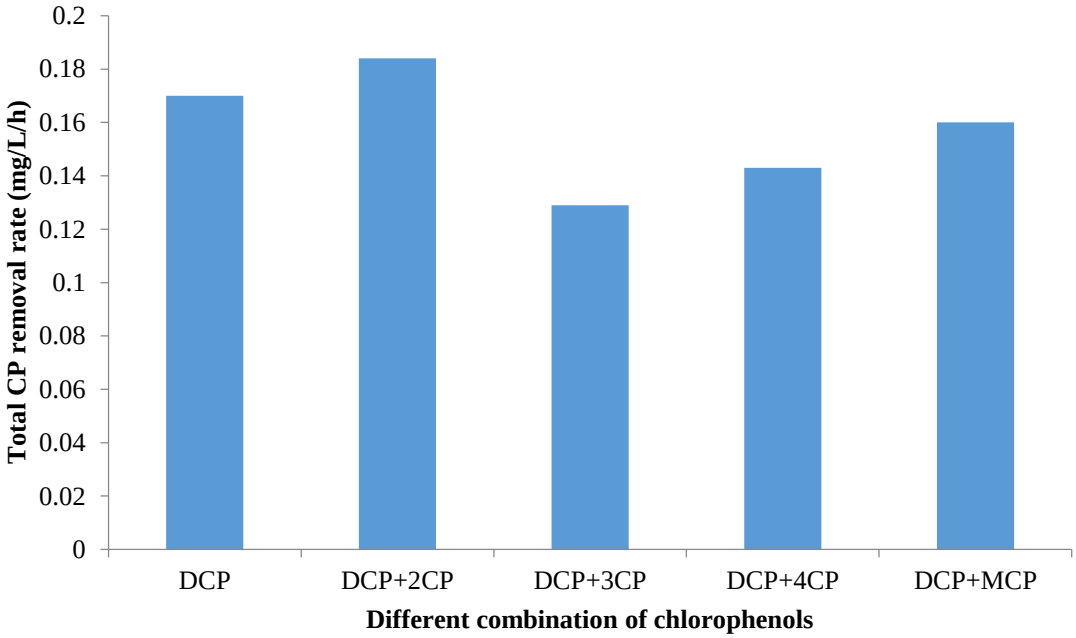


Figure 4.61: Total chlorophenol removal rate obtained for 2,4-DCP and MCPs during the cometabolic study by *P. aeruginosa* GF.

Summary:

This section discusses the summary of three parts 4.2, 4.3 and 4.4 i.e. biodegradation of chlorophenols by single pure culture.

- Three different pure strains *Bacillus endophyticus* CP1R, *Kocuria rhizophila* 11Y and *Pseudomonas aeruginosa* GF were successfully isolated that have potential for biodegradation of chlorophenols.
- All the three strains were able to tolerate and utilize high concentration of 2-CP and 2,4-DCP efficiently. However they have shown minimum tolerance and degradation of 3-CP and 4-CP up to low concentration only. All the three strains were not able to utilize 2,4,6-TCP and PCP individually as single substrate and in cometabolism also. This shows the acclimation to particular compounds has important effect on microorganism.
- RSM has proven effective in parameter optimization and significantly improve the degradation and tolerance capacity of the isolates for 2,4-DCP with minimal inhibitory effects.
- The biodegradation kinetics parameters for 2-CP, 3-CP, 4-CP, and 2,4-DCP, was obtained using the Andrews substrate inhibition model for all three strains. Table summarize the biodegradation kinetic parameters for all three strains for comparison purpose.
- Cometabolic study of 2,4-DCP with three MCPs shows that all the three isolates have able to utilize 2,4-DCP in the presence of all three MCPs. This shows the great prospect of the strains for *in-situ* bioremediation purpose.
- In the presence of 2-CP, the biodegradation of 2,4-DCP and total chlorophenol removal rate increased significantly as compared to 3-CP and 4-CP. The 2-CP is less toxic and easily degradable compound than 2,4-DCP and also has the structural similarity. Here microbes utilize the 2-CP first and contributes for biomass production that leads to higher chlorophenols removal rate. However in case of *P. aeruginosa* GF strain, the isolate has shown higher degradation of 2,4-DCP than 2-CP which is different strategy than other two strains.
- All three strains were able to utilize 2,4-DCP (Higher toxic) in the presence of 3-CP and 4-CP (low toxic) but not able to utilize 3-CP and 4-CP during cometabolism. The possible reason was previously mentioned that the microorganisms utilize different bio-energetic processes for degradation of compounds depending on their toxicity. Also, as mentioned earlier that acclimation has great effect on microorganism. All the thee strains were acclimated to 2,4-DCP and so expresses the enzyme that are responsible for 2-CP and 2,4-DCP degradation only.

Comparison of kinetic parameters for degradation of chlorophenols by all three individual pure culture

	2,4-DCP			2-CP			3-CP			4-CP		
	R_m	K_s	K_i	R_m	K_s	K_i	R_m	K_s	K_i	R_m	K_s	K_i
B. endophyticus CP1R	1.53	711.5	-	1.43	575.8	-	0.123	1	13.08	0.118	20.18	251.9
K. rhizophila 11Y	1.17	568.1	215.63	1.25	289	-	0.384	145.5	12.8	0.8	336.5	23.8
P. aeruginosa GF	2.03	1145	1340.8	1.56	524.34	667.55	0.06	15.4	244.5	0.117	48.47	1251

R_m = maximum removal rate (mg/L/h); *Ks* = haft saturation constant (mg/L); K_i = substrate inhibition constant (mg/L)

4.5.Biodegradation of chlorophenols by defined mixed microbial consortium

In the present section, the biodegradation of chlorophenols including 2-CP, 3-CP, 4-CP, 2,4-DCP, 2,4,5-TCP and PCP by the defined mixed microbial consortium was studied. The effect of cometabolism of lower chlorophenols i.e. monochlorophenols on biodegradation of higher chlorinated phenols such as 2,4-DCP, 2,4,6-TCP and PCP has been evaluated. Also, the presence of metabolite during biodegradation has been analyzed to predict the degradation pathway of the chlorophenols by the mixed consortium.

As it was reported in the previous section that none of the pure isolated cultures was able to degrade the 3-CP, 4-CP, 2,4,6-TCP and PCP alone. But when they were mixed to form a consortium, they showed great efficiency to degrade 3-CP, 4-CP and 2,4,6-TCP. So, in the present chapter, emphasize was given on the biodegradation of 3-CP, 4-CP, 2,4,6-TCP and PCP by the defined mixed consortium.

4.5.1. Biodegradation of monochlorophenols

Biodegradation of 2-CP, 3-CP, and 4-CP at different initial concentration was carried out by the mixed microbial consortium for 168 h. The biodegradation profile of 2-CP, 3-CP, and 4-CP are shown in figure 4.62, 4.63 and 4.64 respectively. None of the individual bacteria has shown degradation of 3-CP and 4-CP. However, all individual bacterium showed degradation of 2-CP. The mixed consortium has shown a higher tolerance level for 4-CP as compared to 2-CP and 3-CP and but the overall removal rate observed was higher for 2-CP. In case of 2-CP, the mixed consortium has not shown complete biodegradation for initial concentration up to 400 mg/L. The biodegradation obtained was 47, 42, 40, 41, 35 and 25 for initial substrate concentration of 50, 100, 150, 200, 300, 400 mg/L within 168 h. However, the percentage degradation decreased after 300 mg/L, and the overall removal rate was increased up to 300 mg/L. The reason for incomplete degradation of 2-CP by the mixed consortium at lower concentration was not clear.

In case of 3-CP, the microorganisms have completely removed 50 mg/L within 30 h while it has removed 95 and 89% of 100 and 150 mg/L within 120 and 168 h respectively. The microorganisms have removed 67, 24, 14 and 4% of 200, 250, 300 and 400 mg/L of 3-CP respectively within 168 h. After 200 mg/L of 3-CP, the inhibition effect is more persistent which results in decreased removal rate and long lag phase.

In case of 4-CP, the microorganisms have completely removed the 50 and 100 mg/L within 32 and 72 h respectively while it has removed 77% of 150 mg/L within 48 h. While the percentage removal observed for 200, 250, 300 and 400 mg/L were 63, 63, 65 and 58% within 168 h respectively. The inhibition effect is higher at 500 and 600 mg/L of 4-CP for which only 13 and 5.5% degradation occurs within 168 h respectively.The removal rate of 3-CP and 4-CP was decreased and become constant due to inhibition effect caused by the accumulation of metabolites.

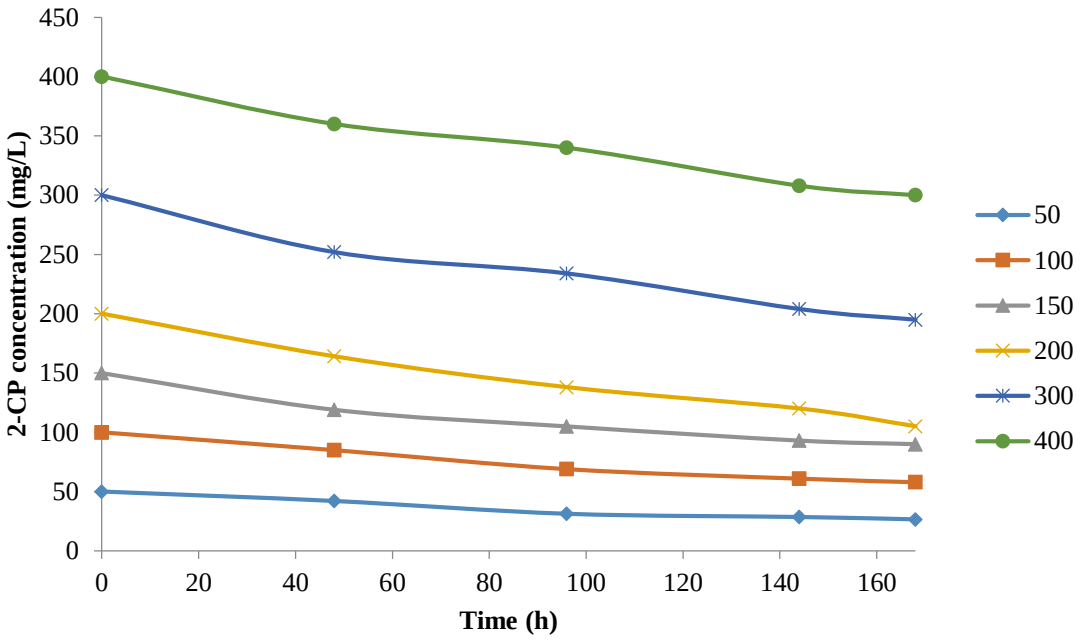


Figure 4.62: Biodegradation profile of 2-CP with time at different initial substrate concentrations.

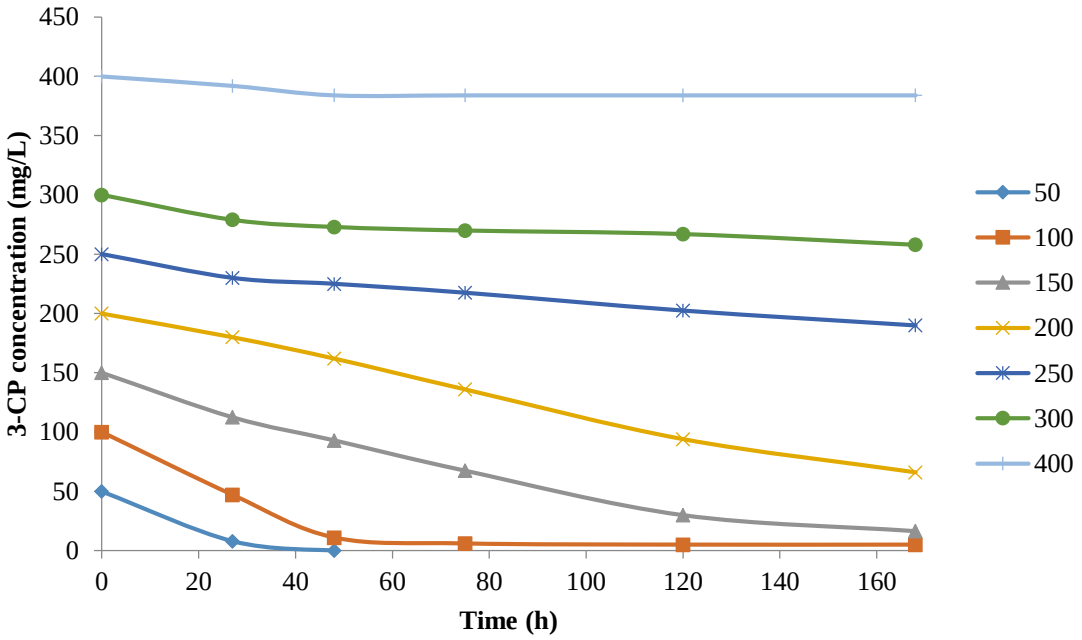


Figure 4.63: Biodegradation profile of 3-CP with time at different initial substrate concentrations.

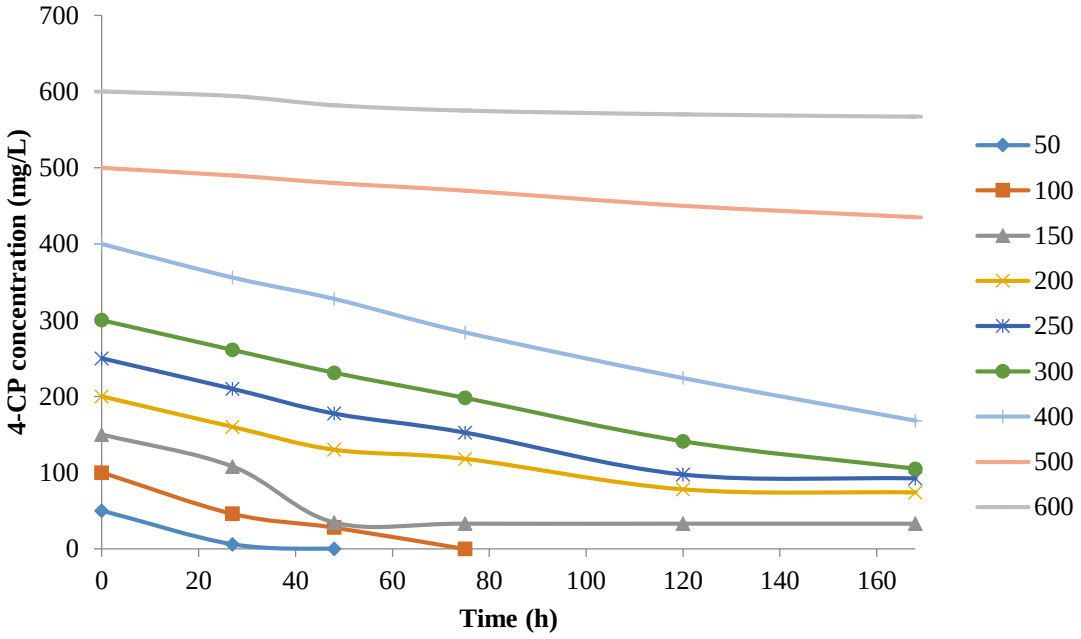


Figure 4.64: Biodegradation profile of 4-CP with time at different initial substrate concentrations.

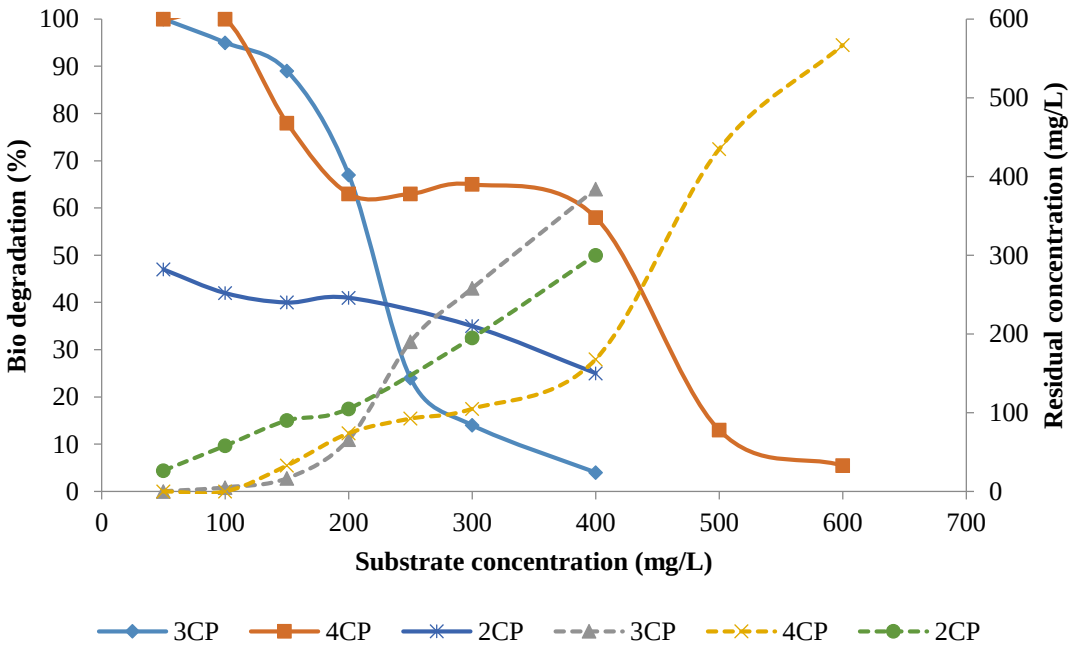


Figure 4.65: Effect of initial MCPs concentration on biodegradation (%) (Solid line) and residual MCPs concentration (Dotted line).

Figure 4.65 shows the effect of initial substrate concentration on the percent biodegradation and final residual concentration of MCP. The biodegradation (%) observed was

higher for 3-CP at low concentration compare to 4-CP, which diminished greatly at a higher concentration. While, the biodegradation (%) observed was higher for 2-CP and 4-CP at high concentration. Figure 4.66 shows the effect of initial substrate concentration on the removal rate of MCPs. For 4-CP, the removal rate increased with substrate concentration up to 400 mg/L and then decreased for 500 and 600 mg/L initial substrate concentration. For 3-CP, the removal rate increased with substrate concentration up to 150 mg/L and then decreased afterward showing the inhibition effect on microorganism at a higher concentration. While for 2-CP, the removal rate was increased up to 300 mg/L and then decreased slightly at 400 mg/L. For 3-CP, the inhibition stage was reached at a lower concentration as compared to 2-CP and 4-CP. The toxicity of the MCP is increased in the order of 2CP<4CP<3CP. The removal of chloride residue at *ortho* position requires less energy while the *Meta* position is more energy demanding due to steric hindrance, indicating the higher toxicity of 3-CP as compare to other MCP [224].

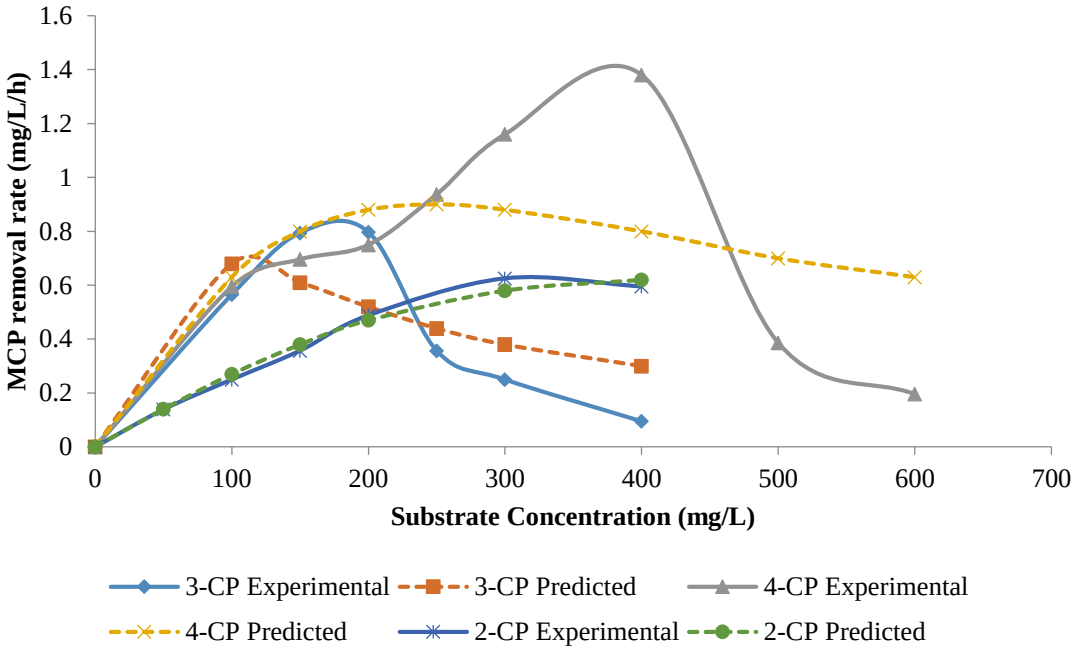


Figure 4.66: Effect of initial substrate concentration on the MCPs removal rate.

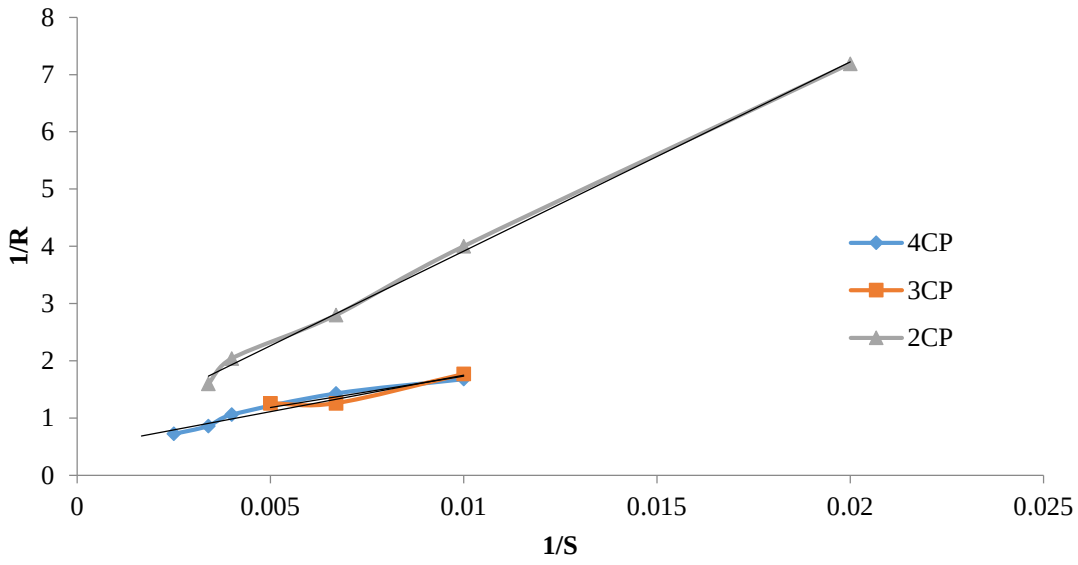


Figure 4.67: Double reciprocal plot of*1/R* v/s *1/S* for MCPs.

Table 4.18 summarizes the biodegradation kinetic parameters for MCP calculated using Andrew's substrate inhibition model (Equation 3.1). The double reciprocal plot between removal rate and substrate concentration has shown in figure 4.67. The maximum removal rate obtained was 2.78, 1.82, and 0.91 mg CP/L/h for 2-CP, 4-CP and 3-CP respectively. The inhibition constant is an important bio-kinetic parameter that determines the inhibition effect of the compound in a microorganism. A higher the inhibition constant, A lower the inhibition effect on the microorganism. The inhibition constant (K_i) obtained for MCP was 1061.6, 323.2, 189.46 mg/L for 2-CP, 4-CP and 3-CP respectively. The higher inhibition constant for 2-CP indicates the less inhibition effect on microorganism compare to 3-CP and 4-CP. 3-CP has the lowest value for inhibition constant indicating higher toxicity on the mixed consortium. The half-saturation constant (*Ks*) obtained for 2-CP, 3-CP and 4-CP was 956, 46.57, and 225.68 respectively. The lower value of half-saturation constant (*Ks*) for 3-CP indicates that the maximum removal rate was achieved at lower concentration compare to 2-CP and 4-CP as shown in figure 4.66. The kinetic constants obtained for 3-CP and 4-CP were in accordance with the literature. The critical substrate concentration (Equation3.5), after which removal rate decreases, obtained was 1007.41, 93.9 and 270 mg/L for 2-CP, 3-CP, and 4-CP respectively.

Table 4.18: Biodegradation kinetic constants obtained for 3-CP and 4-CP by the defined mixed consortium using Andrew's model.

Compounds	**K_S (mg/L)**	**K_i (mg/L)**	**R_m (mg/L/h)**	**R^2**
2-CP	956	1061.6	2.78	0.99
3-CP	46.57	189.46	0.91	0.93
4-CP	225.68	323.2	1.82	0.95

4.5.1. Metabolites

HPLC and spectroscopic analysis showed the presence of different metabolites as the degradation progresses. There were no intermediate products detected during the degradation of 2-CP indicating the complete degradation. During the biodegradation of both 3-CP and 4-CP, one characteristic peak at 251-253 nm was observed. According the literature this peak was related to 2-chloromaleylacetate [235]. This peak was observed as degradation progress and at higher concentration of 3-CP and 4-CP. At lower concentration of 3-CP and 4-CP, this peak was not detected. Figure 4.68 shows the spectrophotometric analysis of 4-CP (50 mg/L) biodegradation by the mixed consortium. From the figure, it was observed that as degradation progresses, a new peak at 253 nm appears. This peak for 2-chloromaleylacetate was disappeared at the end of the degradation indicating the complete mineralization of the MCPs. In case of 4-CP, at higher concentration, the absorbance at 365-370 nm shows the presence of trace amount of 2-hydroxymucnic semialdehyde.

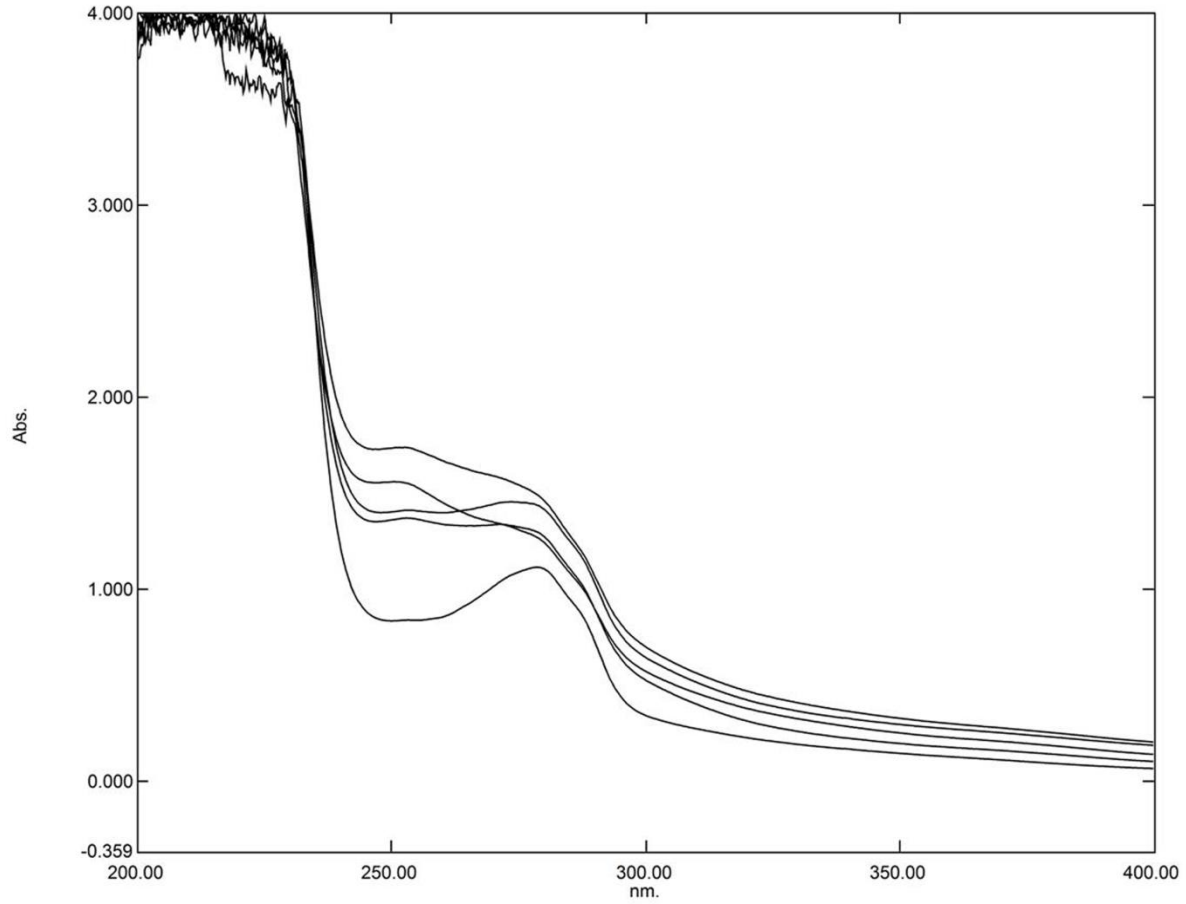


Figure 4.68: Spectrophotometric analysis of biodegradation of 4-CP (50 mg/L by the mixed consortium. The absorbance of 4-CP was a 279 nm and the second appearing at 253 nm related to 2-chloromaleylacetate as the degradation progress.

4.5.2. Co-metabolic study of MCPs and 2,4-DCP

The co-metabolic study was carried out to analyze biodegradation of 2,4-DCP (DCP) in the presence of MCPs by the defined mixed microbial consortium. The co-metabolic study was performed in 250 mL Erlenmeyer with 50 mL mineral salt medium. A different combination of DCP and MCPs added to the flask as mentioned in the table 4.19. Samples were withdrawn at a regular interval for HPLC analysis of residual chlorophenol concentration.

Table 4.19: A different combination of 2,4-DCP and MCPs used in the co-metabolic study by the defined mixed consortium.

Compounds	DCP (mg/L)	2CP (mg/L)	3CP (mg/L)	4CP (mg/L)	Total CP (mg/L)
DCP	50	-	-	-	50
DCP+2CP	50	25	-	-	100
DCP+3CP	50	-	25	-	100
DCP+4CP	50	-	-	25	100
DCP+2CP+3CP	50	25	25	-	100
DCP+2CP+4CP	50	25	-	25	100
DCP+3CP+4CP	50	-	25	25	100
DCP+2CP+3CP+4CP	50	17	17	17	103

The biodegradation of 2,4-DCP (DCP for further discussion) in the presence of three different MCPs, alone and in the mixture, was analyzed, and the results were shown in figure 4.69. The total chlorophenol removal rate for a different mixture of chlorophenols and biomass (maximum OD at 600nm) observed are shown in figure 4.70 and 4.71 respectively.

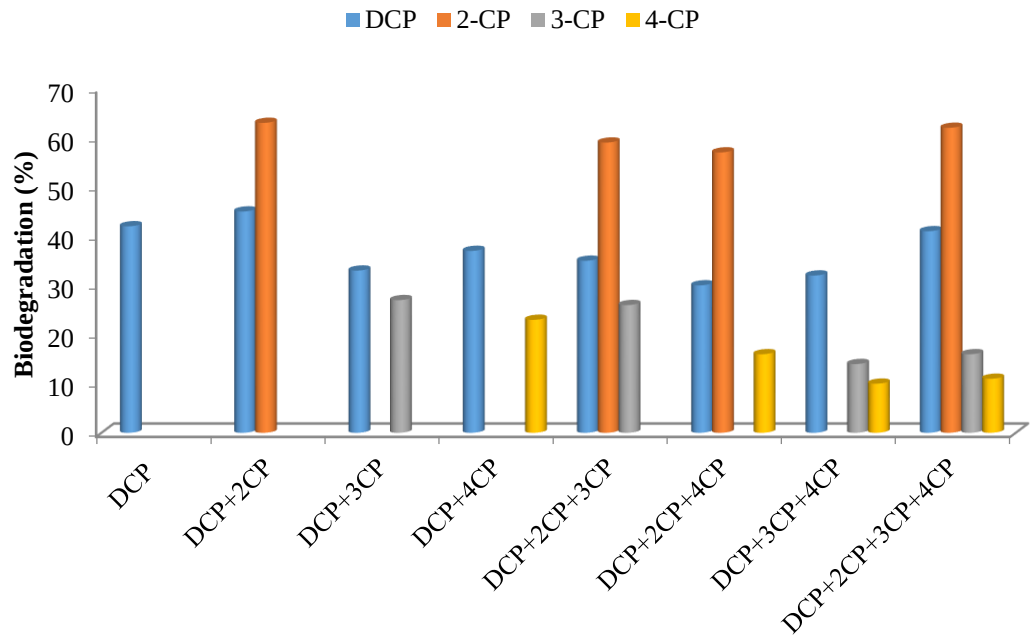


Figure 4.69: Biodegradation (%) obtained for 2,4-DCP and MCPs during the co-metabolic study.

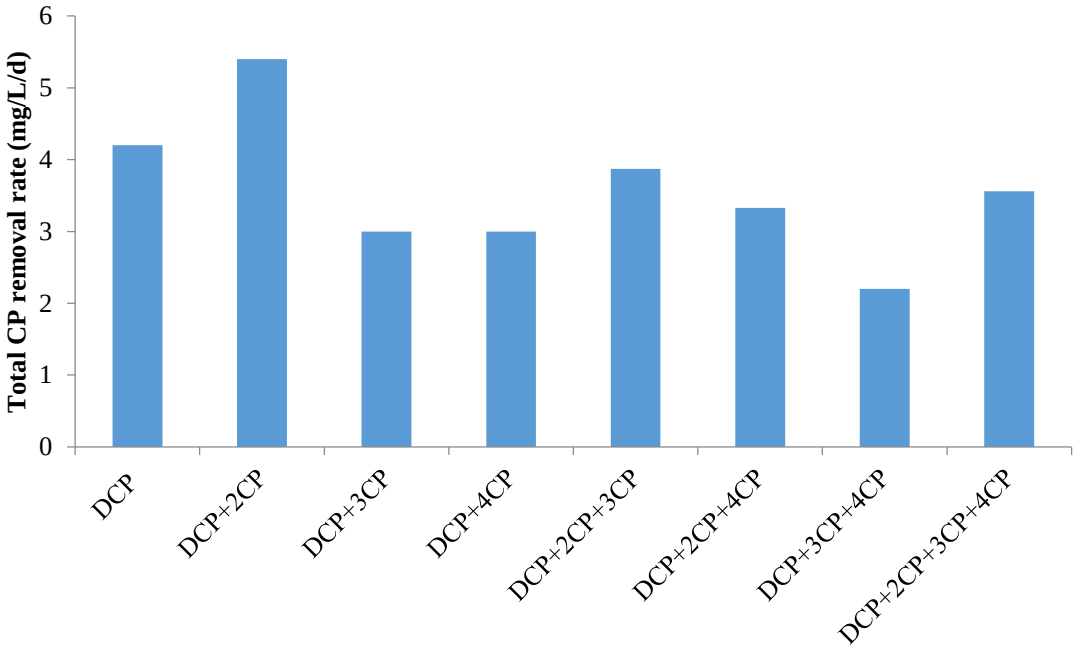


Figure 4.70: Total chlorophenol removal rate obtained for 2,4-DCP and MCPs during the co-metabolic study.

The mixed consortium showed 42% degradation of DCP alone within 240 h. In binary mixture with three different MCP, the mixed consortium showed 45, 33, 37% degradation of DCP in the presence of 2-CP, 3-CP and 4-CP respectively. While the degradation obtained for 2-CP, 3-CP and 4-CP was 63, 27 and 23% respectively. The biodegradation of 2,4-DCP in the presence of 2-CP was observed to increased and that of observed to decreased in the presence of 3-CP and 4-CP. However, the total chlorophenol degradation was higher for 2CP+DCP mixture (54%) compare to DCP alone (42%) and other binary mixture i.e. 3CP+DCP and 4CP+DCP (30% in both cases).

In a tertiary mixture, the highest biodegradation obtained for DCP was 35% in the presence of 2CP+3CP. The biodegradation of DCP was decreased to 30 and 32% in the presence of other binary mixture of MCP as shown in figure 4.69. In the tertiary mixture, among all three MCP, the removal of 2-CP was highest. While the removal of other two MCP, 3-CP, and 4-CP observed to decrease, compare to 2-CP and DCP. The biodegradation (mg/L/d) observed was in the order of DCP>2CP>3CP>4CP.

In a quaternary mixture of DCP and MCP, the degradation obtained for DCP was 41%, which is higher as compared to the tertiary mixture. While the degradation for MCPs observed was 62, 16, and 11% for 2-CP, 3-CP and 4-CP respectively. The degradation obtained for DCP was nearly equal to that obtained for DCP alone. The biodegradation (mg/L/d) occurred in the order of DCP>2CP>3CP>4CP, which was same as observed in the tertiary mixture. The HPLC chromatogram showed the degradation of a mixture of DCP and MCP in the quaternary mixture has shown in figure 4.72.

In the present study, the total chlorophenol degradation observed was higher in the presence of 2-CP as compare to 3-CP and 4-CP. The toxicity of chlorophenols was inversely proportional to the number of chloride ion substitutions. In the present study, the DCP (higher toxic) had shown higher degradation compare to MCPs (lower toxic). Similar results were also observed during the cometabolism of DCP and MCPs by individual pure strains (chapter 4.2, 4.3 and 4.4). Overall it was observed that the biodegradation of DCP and total chlorophenol degradation rate was increased in the presence of MCP, particularly for 2-CP and 4-CP. This phenomenon could be explained as both 2-CP and 4-CP are the degradation product of the 2,4-DCP [224]. The enzymes expressed in the presence of MCPs are also involved in the removal chloride residue from DCP.

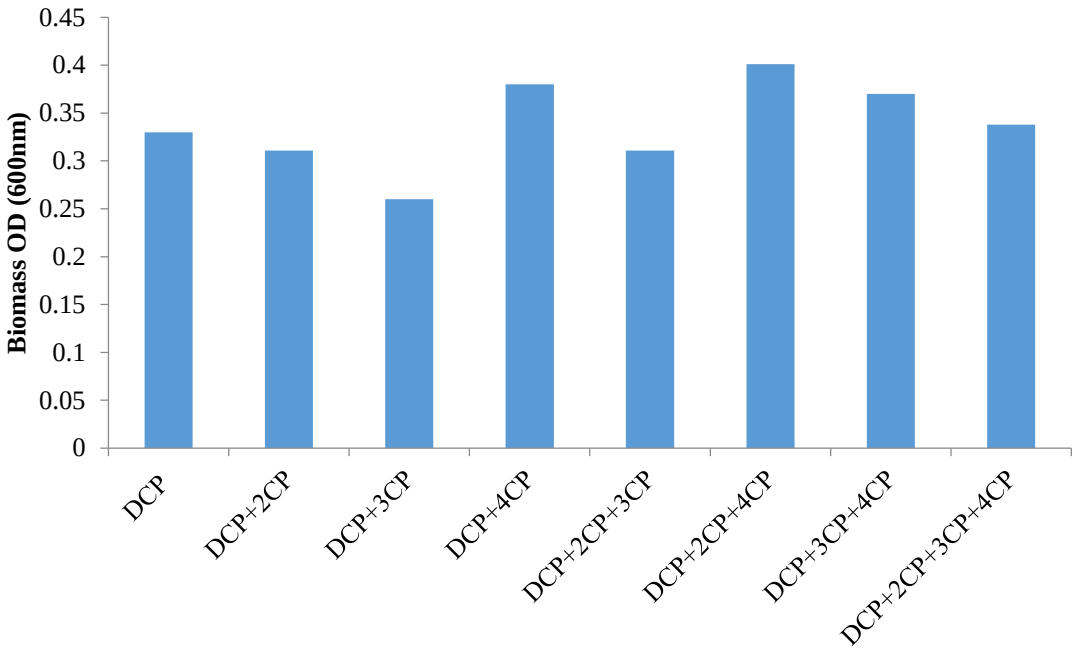


Figure 4.71: Maximum biomass (OD) achieved by the mixed consortium during the co-metabolic study.

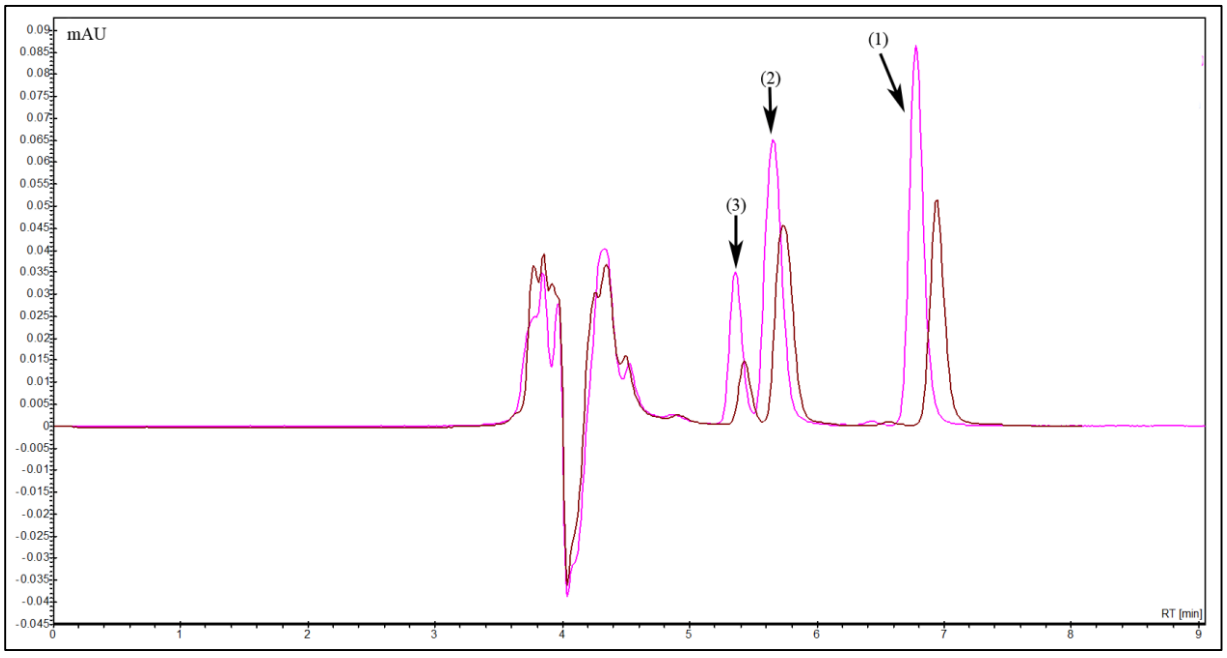


Figure 4.72: HPLC chromatogram for biodegradation of mixture of 2-CP, 3-CP, 4-CP (Total 53 mg/L) and 2,4-DCP (50 mg/L) at 0 and 240 h. The peak identified as 1) 2,4-dichlorophenol, 2) 3-chlorophenol and 4-chlorophenol, 3) 2-chlorophenol. The X-axis shows the retention time (min) and Y-axis shows the absorbance (mAU).

4.5.3. Biodegradation of 2,4,6-TCP by defined mixed consortium

The biodegradation of 2,4,6-TCP by the mixed consortium was performed at different initial substrate concentration for 240 h. All the four isolated strains have not able to degrade 2,4,6-TCP individually. But when all the strains were mixed to form the microbial consortium, they showed good degradation of 2,4,6-TCP. Figure 4.73 and 4.74 shows the biodegradation (%) and residual 2,4,6-TCP concentration at different initial substrate concentration respectively. The biodegradation of 2,4,6-TCP decreased as the substrate concentration increases. The mixed consortium showed 54, 37, 32 and 21% degradation of 25, 50, 75 and 100 mg/L of initial concentration respectively. The rate of degradation was observed to decrease with time due to inhibition effect. The effect of substrate concentration on the removal rate of 2,4,6-TCP is shown in figure 4.75. The removal rate increased up to 75 mg/L of initial substrate concentration, and afterwards it started to decrease. The biokinetic parameters were obtained using the Haldane substrate inhibition model (Equation 3.1). The biokinetic parameters obtained were: maximum removal rate (R_m) 0.3 mg/L/h, half saturation constant (K_s) 95.96 mg/L and substrate inhibition constant (K_i) 75.08 mg/L. The critical substrate concentration (S_{max}or S^*) obtained according to equation 3.5 was 84.8 mg/L, after which the removal rate decreases.

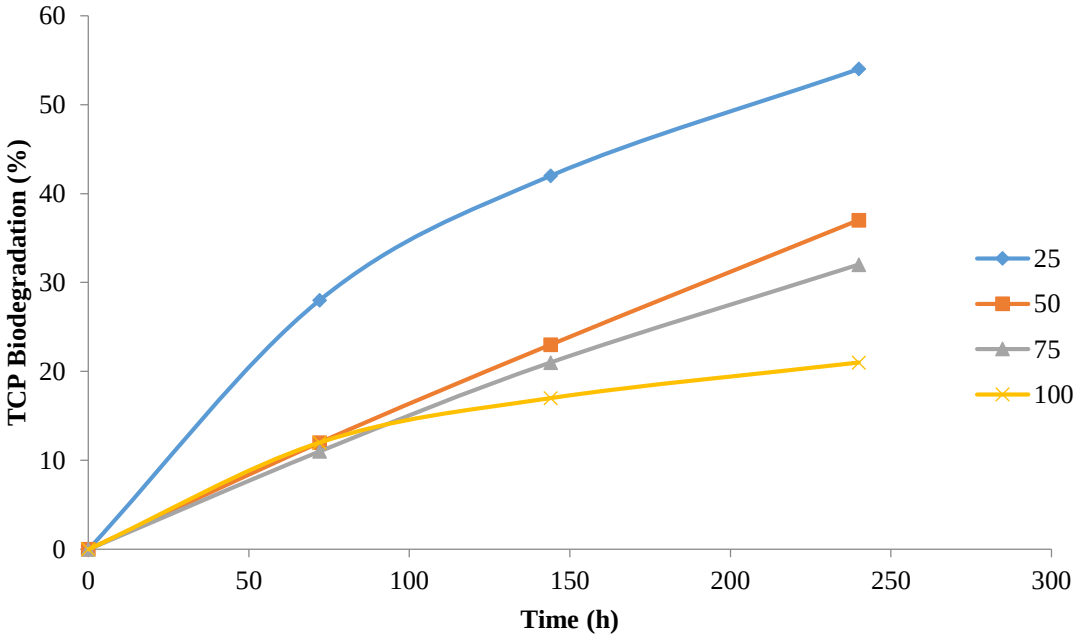


Figure 4.73: Biodegradation of 2,4,6-TCP by the defined mixed microbial consortium.

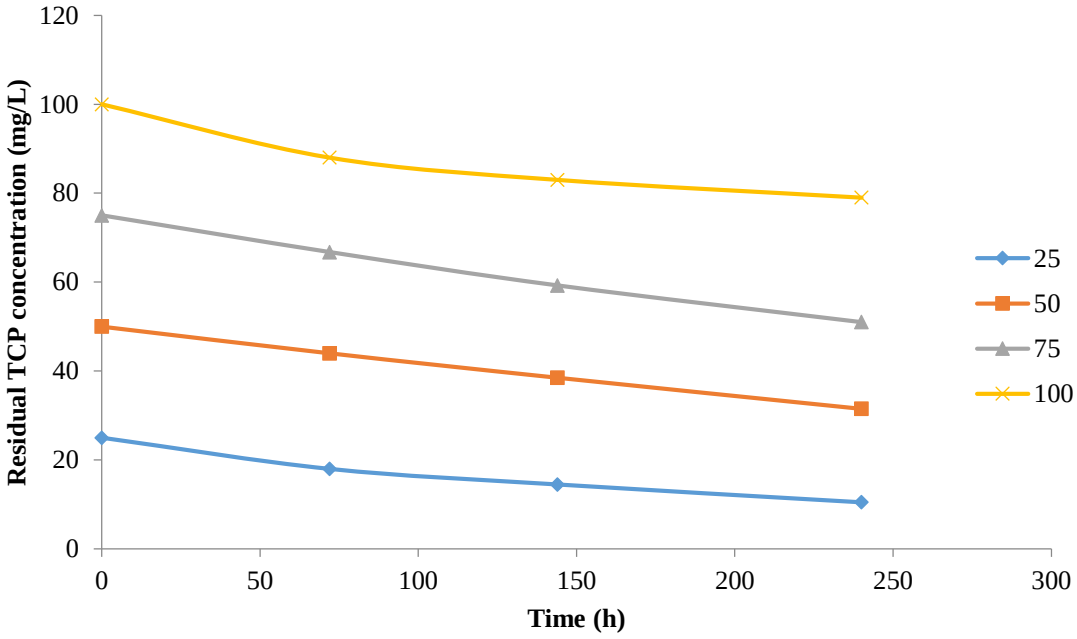


Figure 4.74: The residual concentration of 2,4,6-TCP degradation by the defined mixed consortium at different initial substrate concentration.

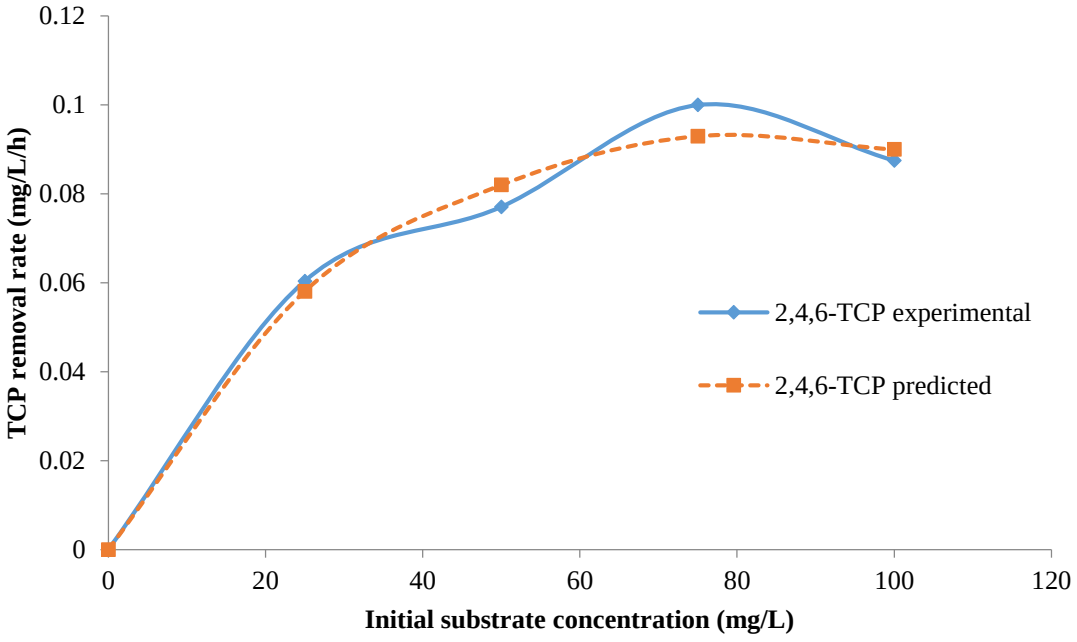


Figure 4.75: Effect of initial 2,4,6-TCP concentration on the removal rate by the mixed consortium.

During the degradation process, several small peaks were observed related to intermediates products. The HPLC chromatogram showing the degradation of 2,4,6-TCP (50 mg/L) at 144 h is presented in figure 4.76. During the degradation process, at 144 h, small peaks related to intermediates are observed. The peak at 6.7 min retention time has been identified as 2,4-dichlorophenol (λ_{max}= 284 nm). Another major peak at retention time of 5.37 min (λ_{max}= 266 nm) was observed. However, the intermediate compound was not identified.

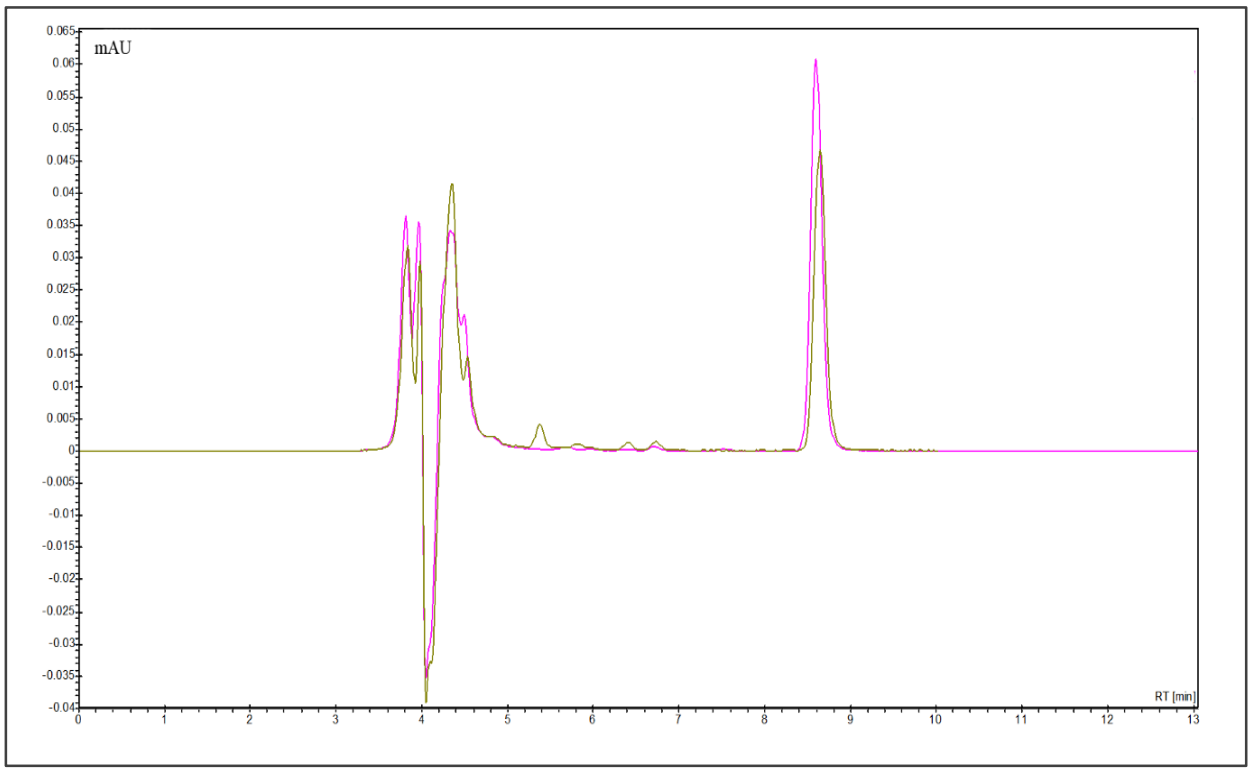


Figure 4.76: HPLC chromatogram showing the degradation of 2,4,6-TCP by the defined mixed consortium at 0 h and 144 h. The retention time for 2,4,6-TCP is 8.6 min.

4.5.4. Cometabolic effect of MCPs and 2,4-DCP on 2,4,6-TCP

The cometabolic study was carried out to analyze biodegradation of 2,4,6-TCP (TCP) in the presence of MCPs and 2,4-DCP (DCP) by the defined mixed microbial consortium. The cometabolic study was performed in 250 mL Erlenmeyer with 50 mL mineral salt medium. A different combination of TCP, DCP and MCPs added to the flask was mentioned in the table 4.20. Samples were withdrawn at a regular interval for HPLC analysis of residual chlorophenol concentration.

Table 4.20: A different combination of chlorophenols used for the cometabolic study of 2,4,6-TCP by the defined mixed consortium.

Compounds	TCP (mg/L)	2CP (mg/L)	3CP (mg/L)	4CP (mg/L)	DCP (mg/L)	Total CP (mg/L)
TCP	50	-	-	-	-	50
TCP+2CP	25	25	-	-	-	50
TCP+3CP	25	-	25	-	-	50
TCP+4CP	25	-	-	25	-	50
TCP+2CP+3CP	25	12.5	12.5	-	-	50
TCP+2CP+4CP	25	12.5	-	12.5	-	50
TCP+3CP+4CP	25	-	12.5	12.5	-	50
TCP+DCP	25	-	-	-	25	50

The biodegradation of chlorophenol mixture was performed in batch for 240 h. The main focus was to study the effect of presence of MCPs and DCP on the degradation of 2,4,6-TCP and the ability of the defined mixed microbial consortium for degradation of a mixture of chlorophenols. MCPs and 2,4-DCP are the degradation products of the 2,4,6-TCP, so their effect on 2,4,6-TCP degradation is important. The total chlorophenol concentration in the medium selected was 50±2 mg/L as shown in Table 4.20. All the four isolated strains have not able to efficiently degrade 3-CP, 4-CP, and 2,4,6-TCP individually, except the strain *Bacillus cereus* 3YS that had shown degradation of 3-CP and 4-CP for lower concentration. But in the consortium, they have shown degradation of 3-CP and 4-CP.

First, the effect of individual MCPs on 2,4,6-TCP degradation was studied. For comparison purpose in mixture study, 50 mg/L of 2,4,6-TCP was taken into consideration. Figure 4.77 summarized the biodegradation of 2,4,6-TCP obtained in the chlorophenol mixture. The mixed consortium showed 35, 31 and 28% degradation of 2,4,6-TCP in the presence of 2-CP, 3-CP and 4-CP respectively which was lower than 2,4,6-TCP alone degradation (37%). The degradation of individual MCPs in the binary mixer obtained was 70, 66 and 46% for 2-CP, 3-CP and 4-CP respectively (Figure 4.77). In the presence of 2-CP, the mixed consortium showed higher degradation compared to 3-CP and 4-CP. 2-CP is easily degradable substrate compared to other to MCPs because the removal of chloride at *meta* and *para* position is more energy demanding process.

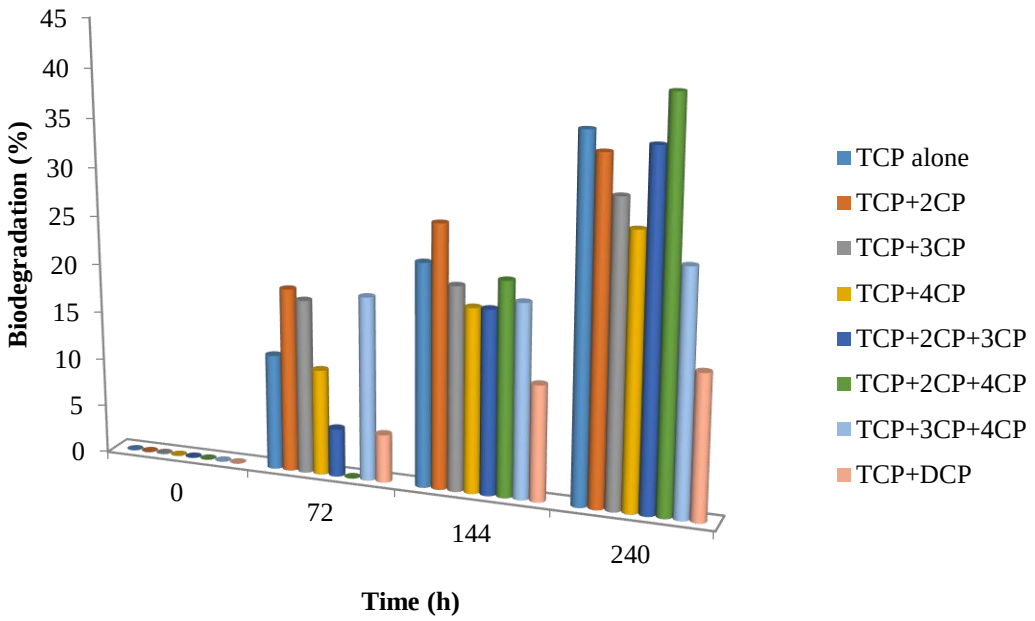


Figure 4.77: Biodegradation of 2,4,6-TCP obtained during the cometabolic study by the defined mixed consortium.

In the next part, the effect of presence of two different MCPs on the degradation of 2,4,6-TCP and total chlorophenol degradation was studied. In tertiary mixture with two MCPs, the degradation obtained for 2,4,6-TCP was 36, 41 and 25% for the combination of 2CP+3CP, 2CP+4CP, and 3CP+4CP respectively (Figure 4.77). The biodegradation of 2,4,6-TCP in the presence of 2-CP+4-CP was increased compared to 2,4,6-TCP alone and in binary mixture with MCPs. Also in the presence of 2-CP+3-CP, the degradation obtained was almost same as that obtained for 2,4,6-TCP alone. The biodegradation of individual MCPs in the binary and tertiary mixture are shown in figure 4.78a, b and c for 2-CP, 3-CP and 4-CP respectively. It was observed that the degradation of 3-CP and 4-CP was increased in the presence of 2-CP. The reason might be the presence of 2-CP which contributes to increased biomass growth that leads to the higher degradation rate. It was also observed that in tertiary mixture with 2CP+3CP and 2CP+4CP, the mixed consortium was first degraded the MCPs and after 72 h, the degradation of 2,4,6-TCP started. This phenomenon was not observed for other combination of chlorophenols. Due to this fact, the total chlorophenol degradation rate and 2,4,6-TCP degradation rate was observed to increase for a tertiary mixture with 2CP+3CP and 2CP+4CP, but it was decreased for 3CP+4CP mixture.

In a binary mixture of 2,4,6-TCP and 2,4-DCP, the mixed consortium showed 15 and 36% degradation for 2,4,6-TCP and 2,4-DCP respectively. In the presence of 2,4-DCP, the 2,4,6-TCP degradation was decreased drastically.

Total chlorophenol biodegradation and total chlorophenol removal rate observed are given in figure 4.79 and 4.80 respectively. The total chlorophenol removal rate was increased in the presence of MCPs compare 2,4,6-TCP alone. Overall in the presence of 2-CP, 3-CP, 2CP+3CP, and 2CP+4CP, the total chlorophenol removal rate observed was higher as compared to 2,4,6-TCP alone and other chlorophenols mixture. While the lowest chlorophenol removal rate observed was in the presence of 2,4-DCP and 4CP. The toxicity of chlorophenols increases in the order of 2,4,6-TCP>2,4-DCP>3-CP>4CP>2-CP. 2,4,6-TCP has three chloride substitution with two chlorides at the *ortho* position and one at the *para* position. The removal of chloride ion at *para* and *meta* site is most difficult and requires more energy as compared to *ortho* site. The lower total chlorophenol degradation observed for the mixture of 2,4,6-TCP with 2,4-DCP and 4-CP was due to the reason that their combination is more toxic than other chlorophenol mixture.

Bae et al., (1997) have isolated two different pure cultures thatwere able to degrade the only selective substrate. *Pseudomonas* sp. TCP114 was able to degrade 2,4,6-TCP and phenol while *Arthrobacter* sp. CPR706 was only able to degrade 4-CP. When two bacteria were mixed, the resulting defined consortium was able to degrade all three chlorophenols simultaneously [20]. Similarly in the present study, the individual strains were not able to degrade 3-CP, 4-CP, and 2,4,6-TCP, but in mixed consortium they have the capability to degrade them. This result show the classic example that the mixed consortium has more potential for degradation of a mixture of chlorophenols and more suited for *in-situ* bioremediation. In the mixed consortium, different microbes act together in a complex manner and their expression changes depending on the substrate structure. In the mixture, biodegradation of chlorophenols compounds depends on the interaction among them, competition, and their structural similarity. Wang et al., (2000) isolated *Pseudomonas* sp. strain 01 and *Pseudomonas* sp. strain 02 that were not capable of degrading 2,4,6-TCP alone. However in the presence of phenol, both strains had shown degradation of 2,4,6-TCP. But the presence of primary substrates such as 2-CP, 3-CP, 4-CP and 2,4-DCP could not assist in the removal of 2,4,6-TCP. The industrial effluent and the environmentally contaminated sites mostly contain the mixture of chlorophenols and other aromatic compounds. In the presence study, the isolated mixed consortium has shown great efficiency for degradation of the mixture of chlorophenols.

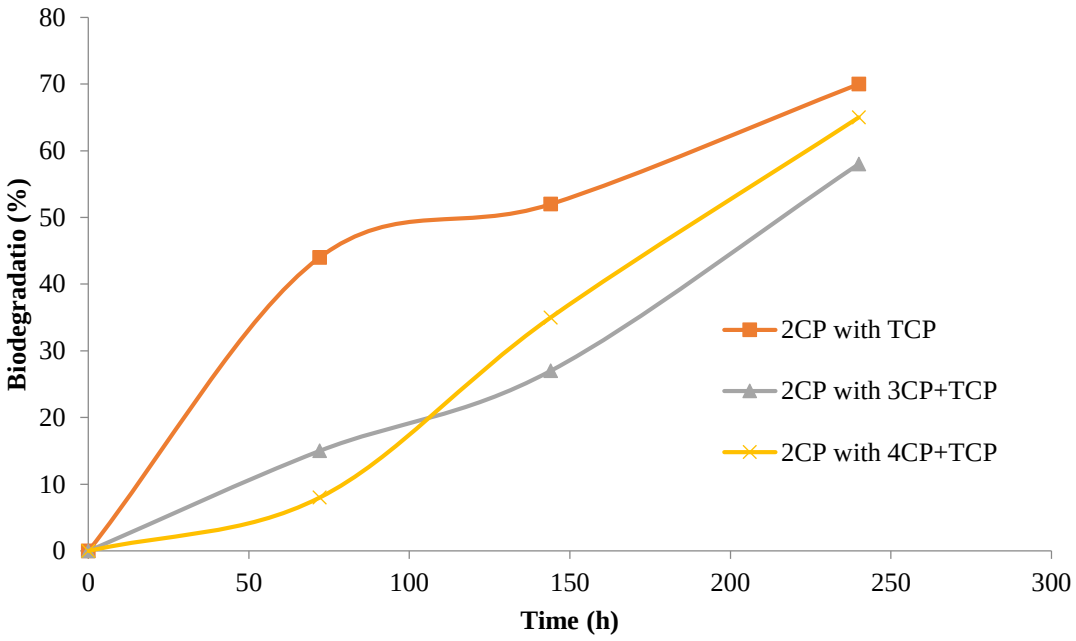


Figure 4.78a: Biodegradation profile of 2-chlorophenol during the cometabolic study of 2,4,6-TCP.

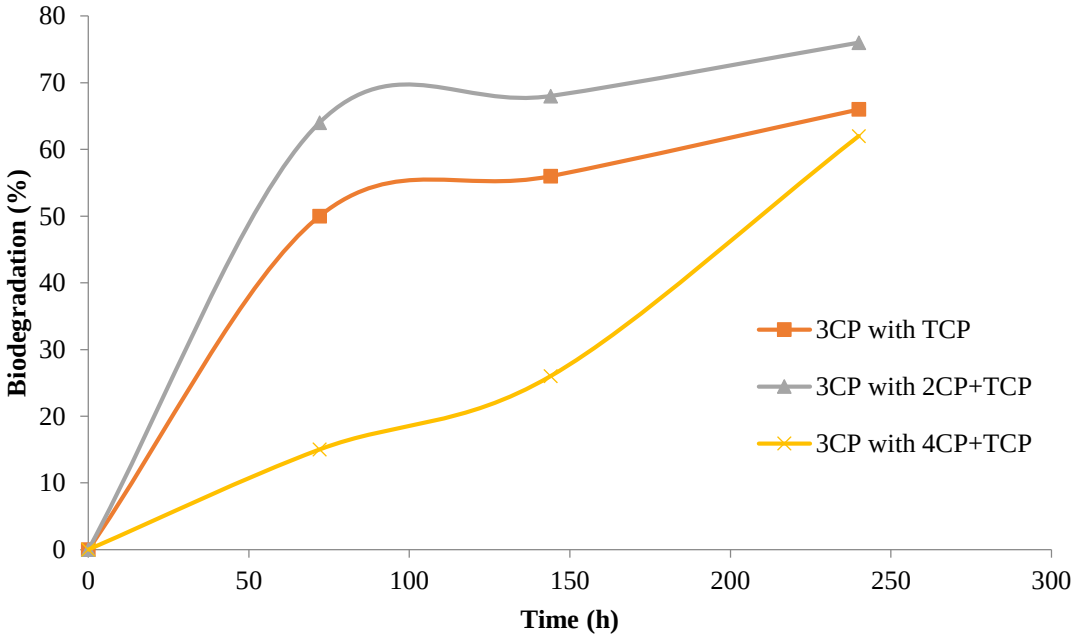


Figure 4.78b: Biodegradation profile of 3-chlorophenol during the cometabolic study of 2,4,6-TCP.

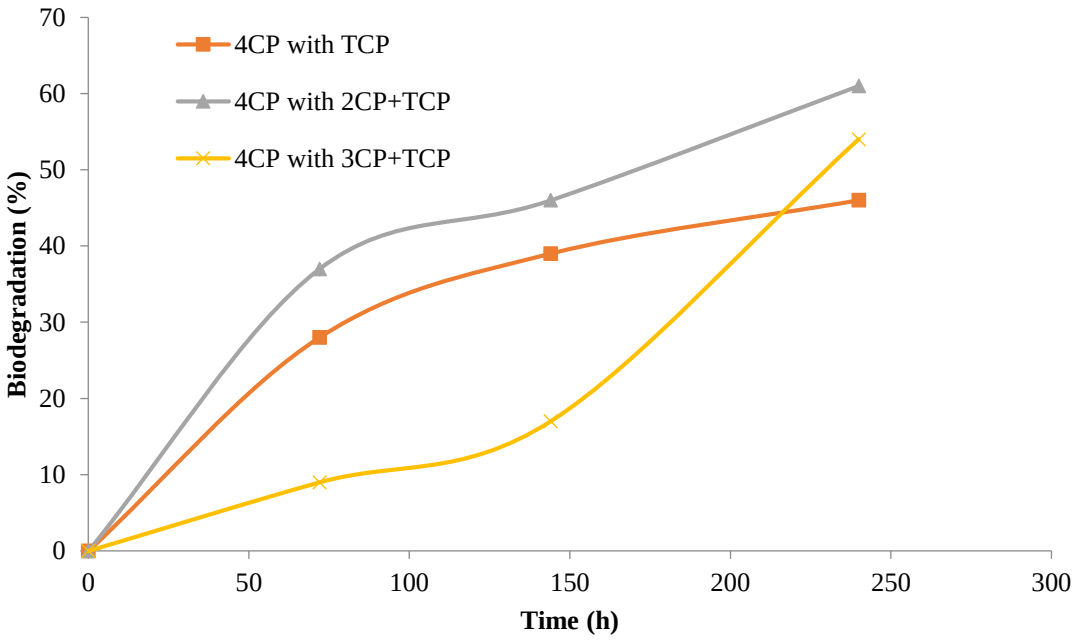


Figure 4.78c: Biodegradation profile of 4-chlorophenol during the cometabolic study of 2,4,6-TCP.

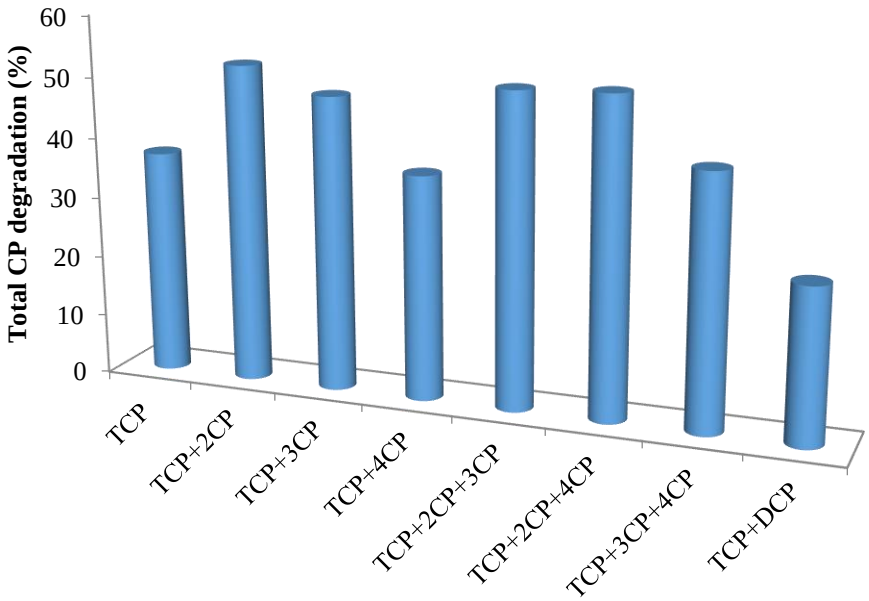


Figure 4.79: Total chlorophenol degradation rate for the cometabolic study of 2,4,6-TCP by the mixed consortium.

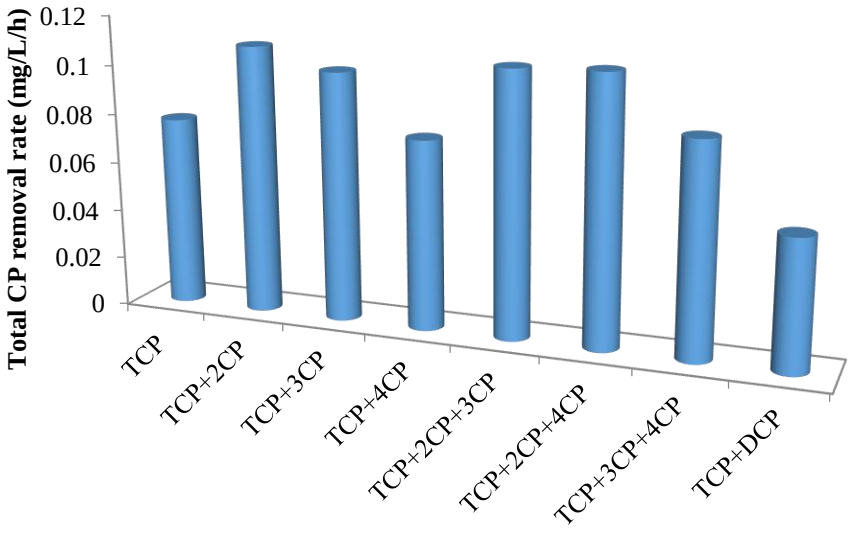


Figure 4.80: Total chlorophenol removal rate obtained for the cometabolic study of 2,4,6-TCP by the mixed consortium.

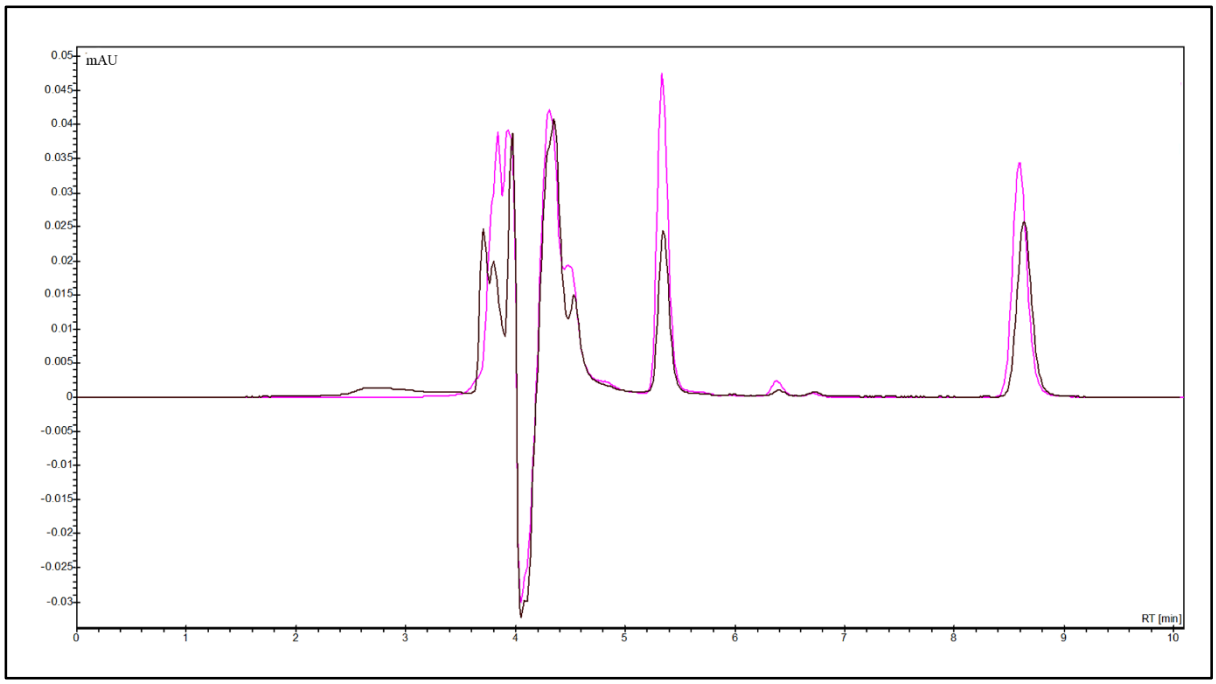


Figure 4.81: HPLC chromatogram showing the degradation of 2,4,6-TCP in the presence of 2-CP by the defined mixed consortium at 0 h and 144 h. The retention time for 2,4,6-TCP and 2-CP are 8.6 and 5.33 min respectively.

4.6. Biodegradation of monochlorophenols and its cometabolism with 2,4-dichlorophenol by microbial consortium (Undefined)

In this section, the ability of undefined mixed microbial consortium was evaluated to utilize chlorophenols. The effect of cometabolism of lower chlorophenols i.e. monochlorophenols on biodegradation of higher chlorinated phenols such as 2,4-DCP, 2,4,6-TCP and PCP was evaluated. Also, the presence of metabolite during biodegradation was analyzed to predict the degradation pathway of the chlorophenols by the mixed consortium. The preparation of undefined mixed consortium was mentioned in chapter 3. In the previous parts, the biodegradation of 2-CP and 2,4-DCP was studied in detailed by pure culture. So in this chapter, emphasize was given on biodegradation of 3-CP, 4-CP, 2,4,6-TCP and PCP with cometabolism in detail.

4.6.1 Biodegradation of 3-CP and 4-CP

Biodegradation of 3-CP and 4-CP by the mixed consortium at different initial concentrations (50 to 600 mg/L) was carried out in batch mode. Biodegradation profile of 3-CP and 4-CP at different initial substrate concentrations are shown in figure 4.82 and 4.83 respectively. In case of 3-CP, 100 and 98% removal was achieved within 48 and 70 h for the initial substrate concentration of 50 and 100 mg/L respectively. While for 200 and 300 mg/L of initial 3-CP concentration, 97 and 44% removal was observed respectively. The inhibition effect was more persistent after 300 mg/L ofinitial 3-CP concentration after which removal rate diminished drastically and only 6.2, 4 and 1% removal observed for the initial substrate concentrations of 400, 500 and 600 mg/L respectively.

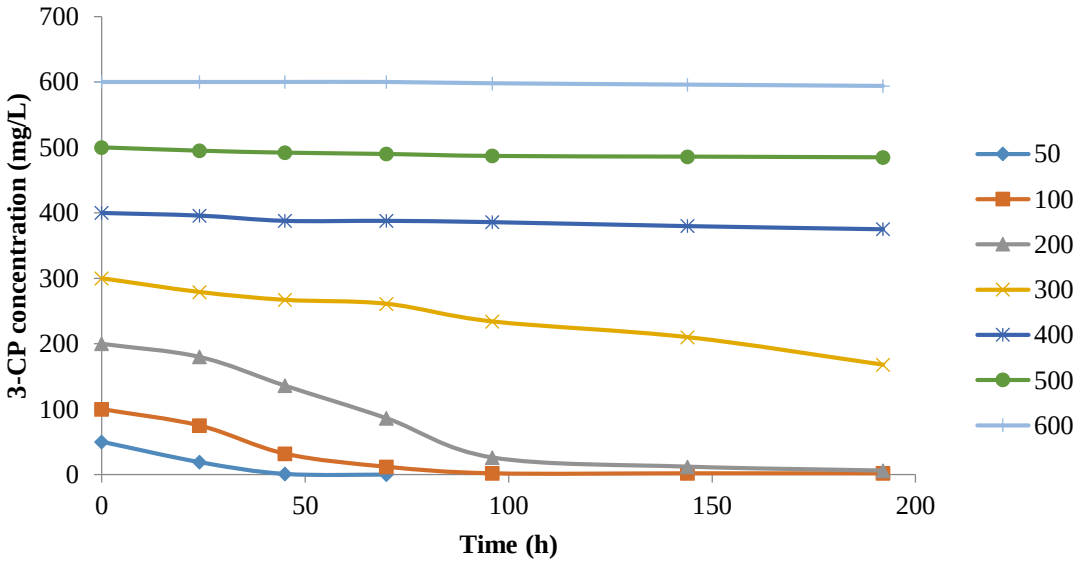


Figure 4.82: Biodegradation profile of 3-CP different initial substrate concentrations by the undefined mixed consortium.

In case of 4-CP, 100% removal was achieved for 50 mg/L initial concentration within 48 h. For initial 4-CP concentrations of 100, 200, 300, 400, 500 and 600 mg/L, the degradation achieved was 90, 80, 79, 75, 67 and 46% respectively. Overall removal efficiency increased up to 400 mg/L of initial 4-CP concentration, and afterward it decreases showing inhibition effect on the microorganism. The overall inhibition effect observed was less in case of 4-CP as compare to 3-CP. However at a lower concentration, up to 200 mg/L, the degradation rate was higher for 3-CP as compared to 4-CP but at higher concentration the degradation rate increases for 4-CP. Also, a diminished biodegradation rate of both 3-CP and 4-CP was observed after a period due to the inhibition effect imposed by the accumulation of metabolites.

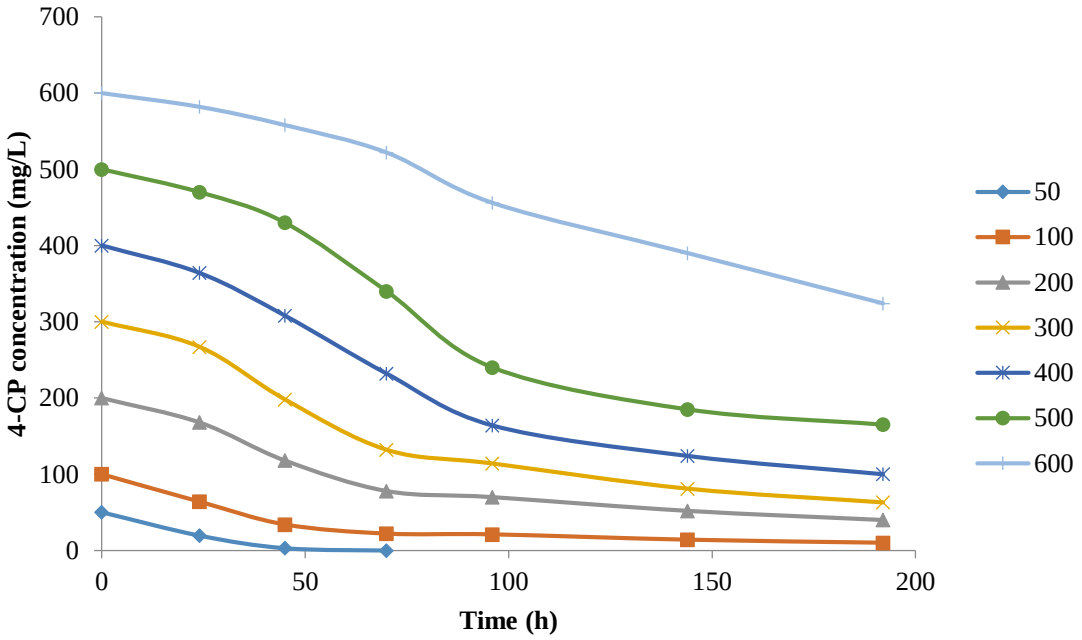


Figure 4.83: Biodegradation profile of 4-CP at different initial substrate concentrations by the undefined mixed consortium.

Figure 4.84 shows the effect of the initial substrate concentrations on percent biodegradation and final residual concentration for 3-CP and 4-CP. In case of 4-CP, the percent biodegradation decreases up to 200 mg/L and then remain nearly constant up to 400 mg/L. While for 3-CP, the percent biodegradation was higher compared to 4-CP for initial concentration up to 200 mg/L, which decreased drastically afterward. Figure 4.85 shows the effect of initial substrate concentrations on the removal rate of 3-CP and 4-CP. For 4-CP, the removal rate increases with substrate concentration up to 400 mg/L and then decreased for 500 and 600 mg/L of initial substrate concentrations. While for 3-CP, the removal rate increases with the substrate concentration up to 200 mg/L and then decreased afterward showing the inhibition effect on microorganism at higher concentration. The substrate inhibition phase was reached at lower concentration for 3-CP due to its higher toxicity as compared to 4-CP.

Table 4.21 summarizes the biokinetic parameters for 3-CP and 4-CP biodegradation calculated using Andrew's substrate inhibition model (Equation 3.1). The maximum removal rate obtained for 3-CP and 4-CP was 1.96 and 6.33 mg CP/L/h. The inhibition constant (K_i) for 4-CP is 1408.8 mg/L, which is greater than 3-CP (K_i =104.12 mg/L) indicating less inhibition effect of 4-CP on microorganisms as compared to 3-CP. The lower value of half-saturation constant (*Ks*) for 3-CP (*Ks* = 47.23 mg/L) shows that it has achieved the maximum removal rate at lower concentration compared to 4-CP (*Ks* = 1262 mg/L). The critical substrate concentration (S_{max} *or* S^*), as per equation 3.5, were determined as 70.12 and 1333.3 mg/L for 3-CP and 4-CP respectively.

Table 4.21. Biodegradation kinetic constants obtained for 3-CP and 4-CP by the undefined mixed consortium using Andrew's model.

Compound	*Ks* (mg/L)	K_i(mg/L)	R_m (mg/L/h)	R^2
3-CP	47.23	104.12	1.96	0.96
4-CP	1262	1408.8	6.33	0.97

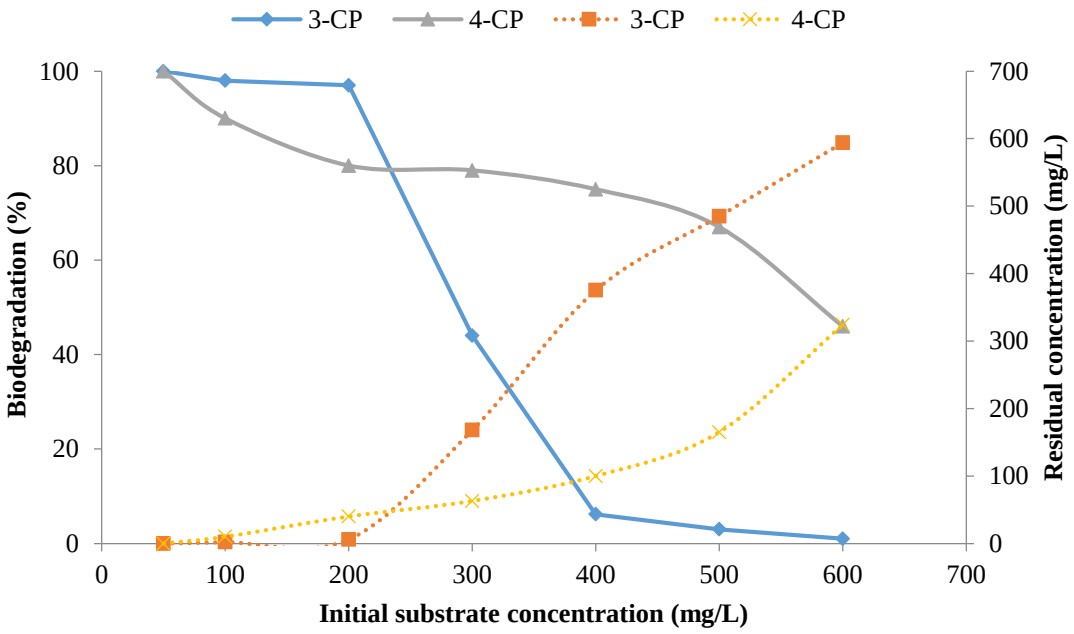


Figure 4.84: Effect of initial substrate concentrations on biodegradation (%) (Solid line) and residual MCPs concentration (Dotted line).

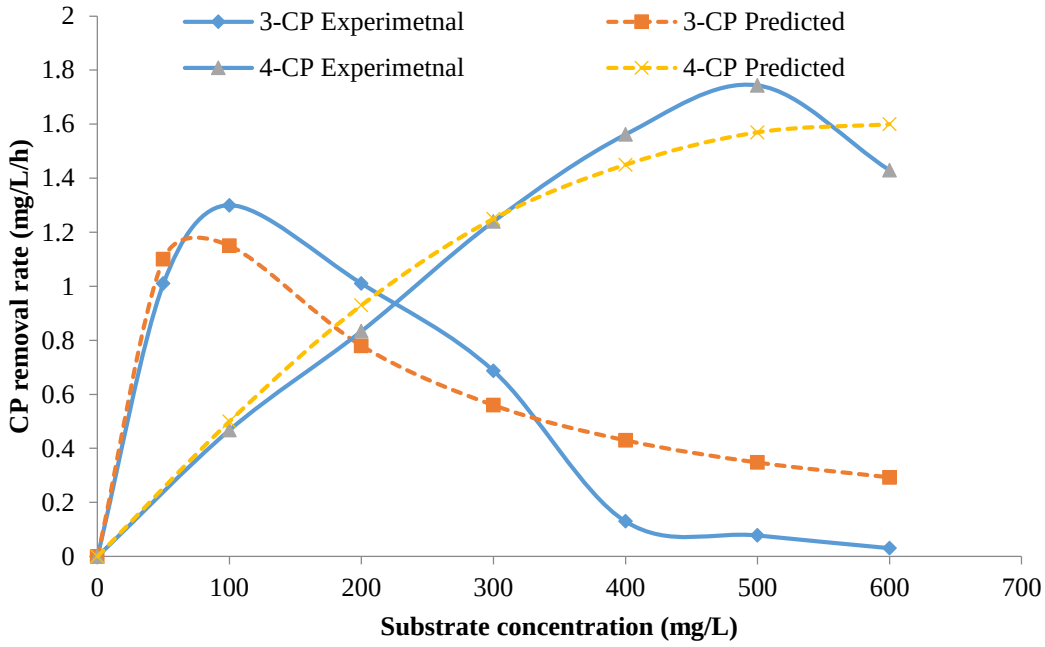


Figure 4.85: Effect of initial substrate concentrations on 3-CP and 4-CP removal rate.

4.6.1.1. Identification of intermediate metabolites

Analysis of intermediate products is important for depicting the biodegradation pathway followed by the mixed microbial consortium. HPLC and spectroscopic analysis showed the presence of different metabolites as the degradation progresses. The analysis of the intermediate products of 4-CP biodegradation by HPLC and spectrophotometric method indicated that it follows the *meta*-cleavage pathway. The calorimetric assay indicated the presence of 4-chlorocatechol (4-CC) in the medium and its concentration was observed to decreased with degradation. The presence of yellow colored 5-chloro-2-hydroxymuconic semialdehyde (5-CHMS) (λmax= 380 nm), the *meta*-cleavage product of 4-CC, was detected during the degradation of 4-CP (Figure 4.86). The degradation of 4-CP via *meta*-cleavage pathway by the mixed consortium and single microorganism have been reported [49, 223]. As the degradation further proceeds, the absorbance peak appeared at 243-247 nm that is a characteristic peak of maleylacetate [43, 230]. The maleylacetate, one of the precursors of TCA cycle, was then degraded via TCA cycle indicating complete mineralization of 4-CP by the consortium.

In case of 3-CP, the calorimetric assays indicated the presence chlorocatechol. The HPLC and spectrophotometric analysis showed the presence of 4-chlorcatechol (4-CC) during the degradation of 3-CP instead of 3-chlorocatechol. This pathway, the transformation of 3-CP to 4-CC, has also been reported earlier [41, 167]. The 4-CC was then further degraded via 5-CHMS (λmax= 380 nm) (Figure 4.87). The 5-CHMS was then converted to maleylacetate (λmax= 245 nm), as mentioned above, which finally enters into the TCA cycle indicating complete mineralization of 3-CP.

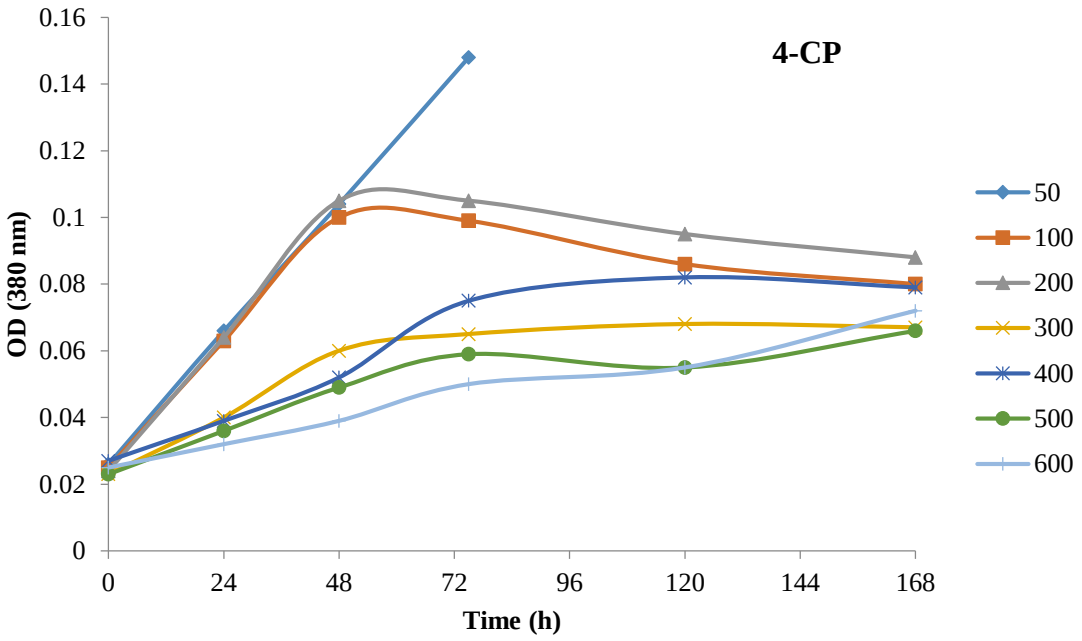


Figure 4.86: The concentration of 5-chloro-2-hydroxymuconic semialdehyde (5-CHMS) during the biodegradation of 4-CP at different initial substrate concentration (λmax = 380nm).

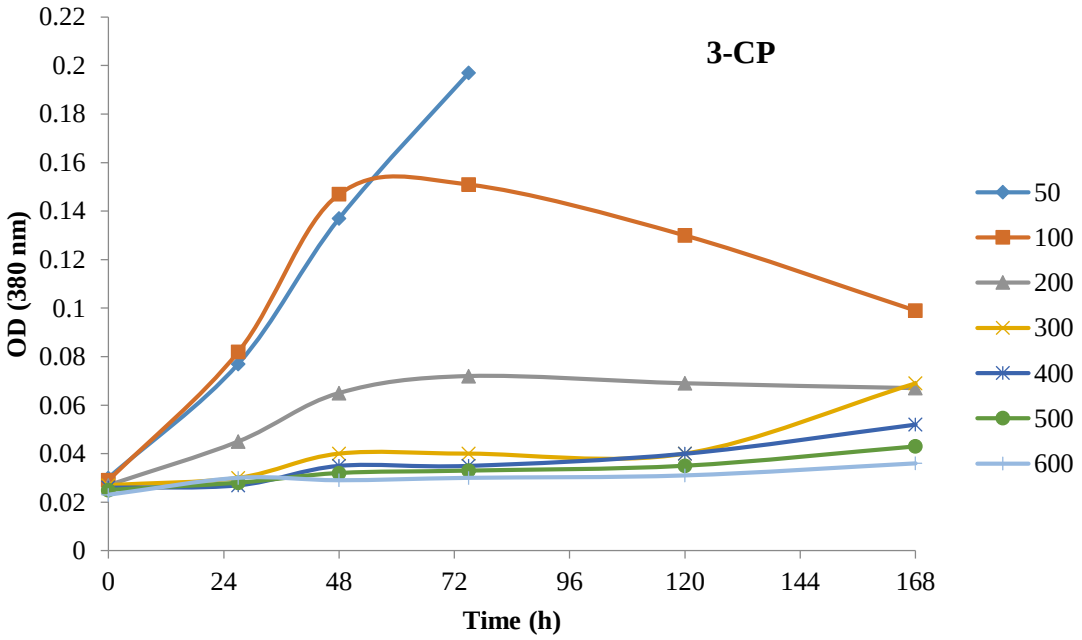


Figure 4.87: The concentration of 5-chloro-2-hydroxymuconic semialdehyde (5-CHMS) during the biodegradation of 3-CP at different initial substrate concentration (λmax = 380nm).

HPLC chromatogram demonstrating the transformation of 3-CP via 4-CC to maleylacetate is shown in figure 4.88. The retention time observed for 3-CP, 4-CC and maleylacetate was 5.6, 4.75 and 3.93 min respectively. Initially, the concentration of 4-CC increased with time and then decreased with the complete degradation of 3-CP, which was converted to maleylacetate. The absorbance peak for maleylacetate increases with the conversion of 4-CC showing the complete degradation of 3-CP (Figure 4.88). The same pathway was also observed for the 4-CP degradation (graph not shown).

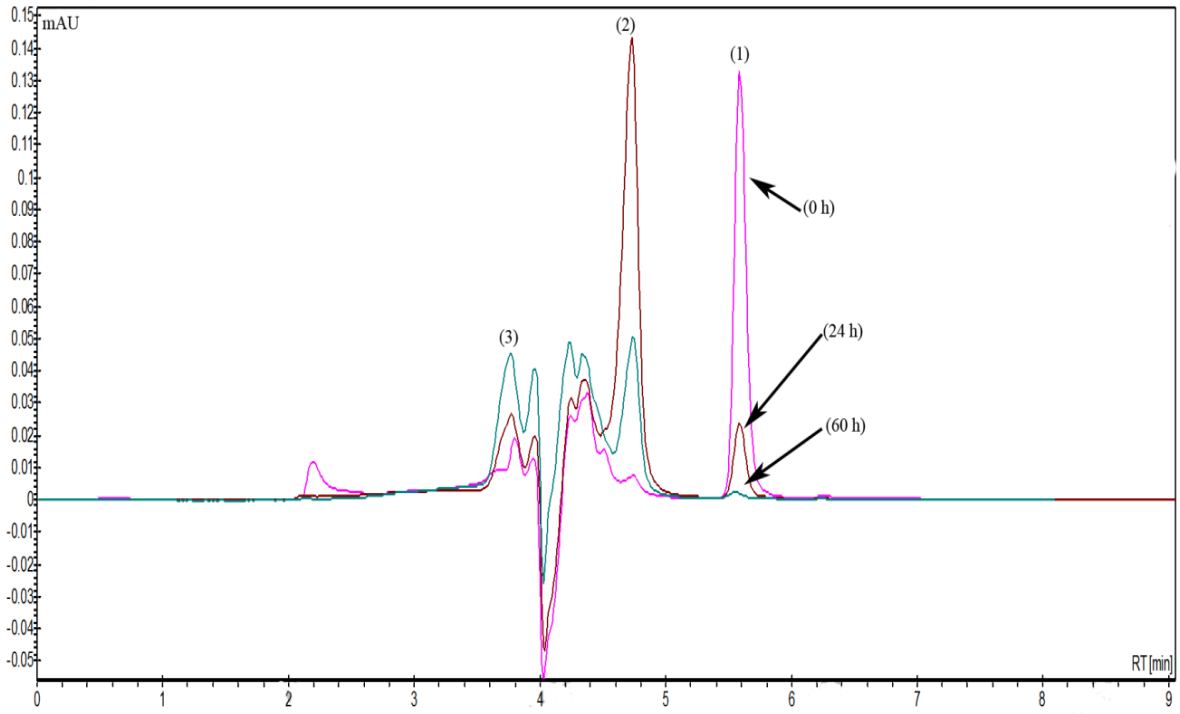


Figure 4.88: HPLC chromatogram showing the biodegradation of 3-CP (50 mg/L) at 0, 24 and 60 h. The peak identified as 1) 3-chlorophenol (RT= 5.6 min), 2) 4-chlorocatechol (RT= 4.75 min), 3) maleylacetate (RT= 3.93 min).

4.6.2 Co-metabolic study of MCPs and 2,4-DCP

The cometabolism of monochlorophenols with 2,4-DCP by the undefined mixed consortium was evaluated. Different combination of 2,4-DCP and MCPs used for cometabolic study was mentioned in table 4.22. The biodegradation of 2,4-DCP (DCP for further discussion) in the presence of three different MCPs, alone and in combination, was analyzed, and the result is shown in figure 4.89.

Table 4.22: A different combination of 2,4-DCP and MCPs used for the cometabolic study by the undefined mixed consortium.

Compound	DCP (mg/L)	2CP (mg/L)	3CP (mg/L)	4CP (mg/L)	Total CP (mg/L)
DCP	25	-	-	-	25
DCP	50	-	-	-	50
DCP	100	-	-	-	100
DCP+2CP	50	25	-	-	100
DCP+3CP	50	-	25	-	100
DCP+4CP	50	-	-	25	100
DCP+2CP+3CP	50	25	25	-	100
DCP+2CP+4CP	50	25	-	25	100
DCP+3CP+4CP	50	-	25	25	100
DCP+2CP+3CP+4CP	50	17	17	17	103

The biodegradation of DCP alone by the mixed consortium achieved 33, 21 and 15% degradation for 25, 50 and 100 mg/L of initial substrate concentrations respectively. The percent biodegradation decreased with increasing DCP concentration, but the overall removal rate (mg/L/d) was observed to increase (Figure 4.89). In a binary mixture of DCP and MCPs, the total chlorophenol degradation observed was 61.5, 38 and 27.5% for 3-CP, 2-CP and 4-CP respectively. The DCP degradation observed was 21% in the presence of 2-CP and 4-CP while it was 24% in the presence of 3-CP which was higher than biodegradation of DCP alone.

In the tertiary mixture, the highest biodegradation for DCP obtained was 28% in combination with 2-CP and 3-CP. While for the other two combination of MCPs, the DCP degradation obtained was 20 and 21% as shown in figure 4.89. The biodegradation of DCP was observed to be highest in the presence of 2-CP (-*ortho*) and 3-CP (-*meta*) as compared to DCP alone and all other combinations. In a tertiary mixture, the biodegradation (mg/L/d) of MCP and DCP was observed in the order of 3CP>4CP>2CP>DCP. In the quaternary mixture, the biodegradation of DCP obtained was 25% of the initial substrate concentration that was higher as compared to DCP alone and binary mixture. Here, the biodegradation (mg/L/d) of DCP and MCPs by the mixed consortium takes place in the order of 3CP>4CP>DCP>2CP.

The biodegradation of DCP was observed to increase in the presence of MCPs due to structural similarity and enzyme secretion in the presence of MCPs. The degradation of DCP was found to be highest in the presence of 3-CP and then 4-CP. Total chlorophenol degradation (%) was also observed in the order of (DCP+3CP)> (DCP+3CP+4CP)> (DCP+3CP+4CP+2CP). The toxicity of MCP increases in the order of 2CP<4CP<3CP. But in this study, it was observed that the degradation of 3-CP is higher as compared to 4-CP and 2-CP. The biodegradation of 2-CP alone by the consortium shows the low degradation rate compare to 3-CP and 4-CP (data not shown). The combination of 3CP and DCP in the mixture exhibits more toxicity than other MCPs combinations. But it was observed that in the presence of 3-CP and DCP, the biodegradation rate was higher as compared to other combination of compounds.

Both defined and undefined consortium have shown different or opposite degradation characteristic. Here, the undefined consortium has more potential or enzyme system that act on 3-CP and 4-CP efficiently than 2-CP. While in case of defined consortium, the 2-CP degradation is higher than 3-CP and 4-CP comparatively. During cometabolism study, in case of the defined mixed consortium, the 2-CP (-*ortho* substitute) was contributed to higher total chlorophenol removal and also, increased degradation of high chlorinated compounds (DCP and TCP) as compared to 3-CP and 4-CP. While in case of the undefined mixed consortium, 3-CP (-*meta* substitute) and then 4-CP (-*para* substitute) has given more contribution to total chlorophenol removal and increased degradation of 2,4-DCP as compared to 2-CP.

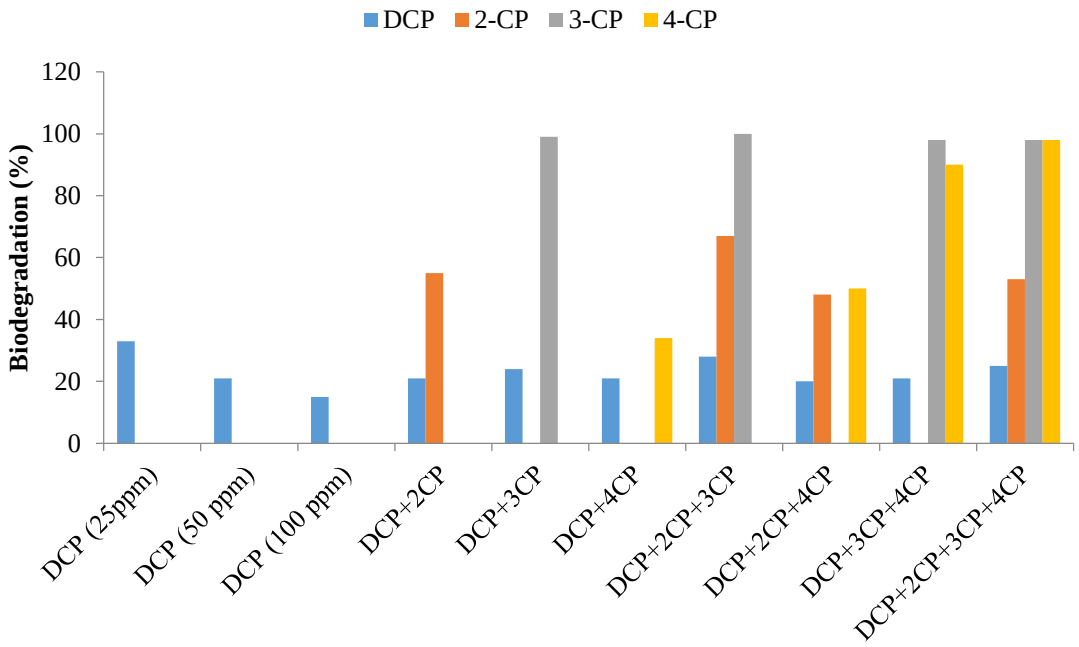


Figure 4.89: Biodegradation (%) obtained for 2,4-DCP and MCPs during the cometabolic study.

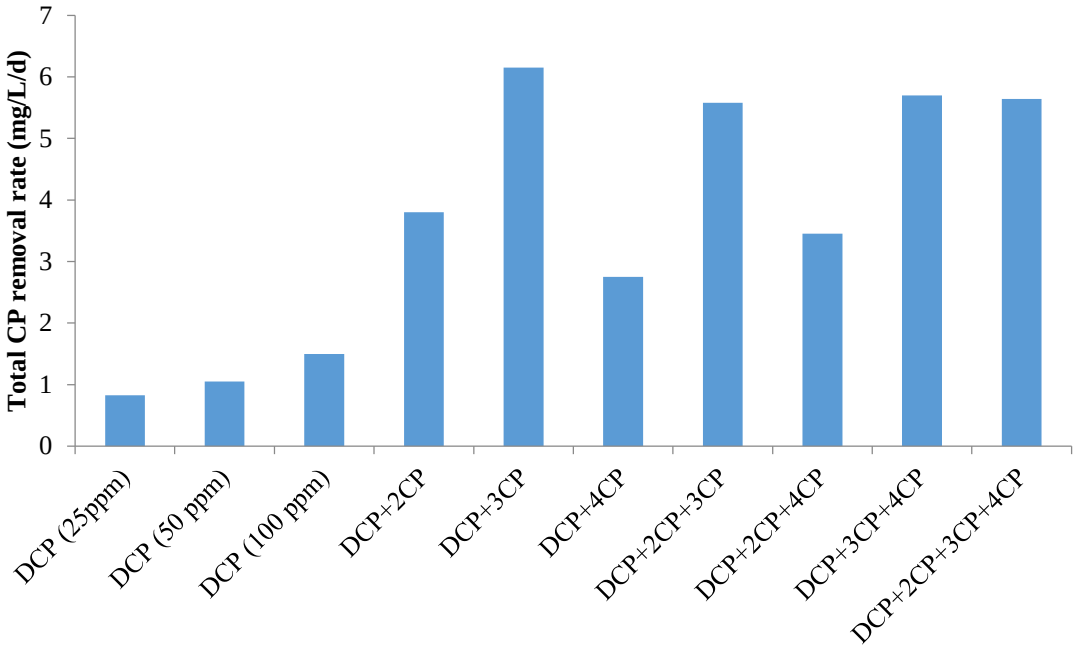


Figure 4.90: Total chlorophenol removal rate obtained for 2,4-DCP and MCPs during the co-metabolic study.

In the present study, the mixed consortium used the different enzyme mechanisms for degradation of MCPs, alone or in the mixture. The degradation of 3-CP and 4-CP both follows the same pathway as mentioned above indicating the involvement or expression of same enzyme systems. The higher degradation of DCP in the presence of 3-CP and 4-CP may be due to the enzyme induced during the degradation of 3-CP and 4-CP. The enzyme expressed during the degradation of 4-CC, the first intermediate of 3-CP and 4-CP, has involved in the degradation of 2,4-DCP due to structural similarity. However, the exact mechanism involved requires further study.

4.6.3 Cometabolism of 2,4,6-TCP

Biodegradation of 2,4,6-TCP and effect of MCPs and 2,4-DCP on its degradation was studied by the undefined mixed consortium. Different concentration of chlorophenols used in the study was mentioned in the table 4.23.

Table 4.23: A different combination of chlorophenols used for the cometabolic study of 2,4,6-TCP by the undefined mixed consortium.

Compounds	TCP (mg/L)	2CP (mg/L)	3CP (mg/L)	4CP (mg/L)	DCP (mg/L)	Total CP (mg/L)
TCP	25	-	-	-	-	25
TCP	50	-	-	-	-	50
TCP	75	-	-	-	-	75
TCP	100	-	-	-	-	100
TCP+2CP	25	25	-	-	-	50
TCP+3CP	25	-	25	-	-	50
TCP+4CP	25	-	-	25	-	50
TCP+DCP	25	-	-	-	25	50

The result indicated that the undefined mixed consortium was unable to utilize 2,4,6-TCP alone or in mixture with MCPs and 2,4-DCP. However, during cometabolism it showed the degradation of 2,4-DCP and MCPs. The consortium has shown 98, 100, 81 and 71% degradation of 2-CP, 3-CP, 4-CP and 2,4-DCP in the mixture with 2,4,6-TCP. Compared defined mixed consortium, undefined consortium proven ineffective for degradation of 2,4,6-TCP alone and in mixture even though both mixed consortium were acclimated to 2,4,6-TCP for same period.

Summary:

This section discussed the summary of two parts 4.5 and 4.6 i.e. biodegradation of chlorophenols by the mixed microbial consortium.

- In the present study, both defined and undefined mixed consortium have shown potential for chlorophenols degradation up to higher concentration.
- In case of defined mixed culture, where none of the individual strains were able to utilize 3-CP, 4-CP, 2,4,6-TCP and PCP alone, showed great degradation efficiency when mixed to form the consortium. In the consortium, they interact with each other and their genetic expression changes which leads to degradation of 3-CP, 4-CP, 2,4,6-TCP and even PCP.
- While in case of undefined consortium, it was able to degrade MCPs and 2,4-DCP only but not able to utilize 2,4,6-TCP and PCP alone and also during cometabolism.
- Defined mixed consortium has degraded MCPs via both *ortho* (primarily) and *meta* pathway as evidenced by the presence of metabolites. While, undefined consortium has followed *meta* cleavage pathway for both 3-CP and 4-CP. Also, it has degraded 3-CP via 4-chlorocatechol instead of 3-chlorocatechol, a rare pathway observed in the literature.
- The biodegradation kinetics parameters for 2-CP, 3-CP, 4-CP,2,4-DCP and 2,4,6-TCP were obtained using the Andrews substrate inhibition model for both mixed consortia. The undefined consortium has shown higher removal rate for 3-CP and 4-CP than defined consortium but has shown very low degradation for 2-CP (Table Below).
- Defined mixed consortium has shown significant results during the cometabolic study of 2,4-DCP and 2,4,6-TCP. The presence of 2-CP contributes to increased total chlorophenol and 2,4-DCP degradation rate as compared to the presence of 3-CP and 4-CP. Also, the 2,4-DCP has shown higher degradation compare to MCPs. The biodegradation (mg/L/d) observed was in the order of **DCP>2CP>3CP>4CP**. While in case of 2,4,6-TCP cometabolism, the total chlorophenol removal rate increased in the presence of 2-CP, 3-CP, 2CP+3CP, and 2CP+4CP as compared to 2,4,6-TCP alone and other chlorophenols mixture. While the lowest chlorophenol removal rate observed was in the presence of 2,4-DCP and 4CP. Also, the presence of 2-CP leads to increased degradation of 3-CP and 4-CP. The defined mixed consortium has able to utilize all the chlorophenols compounds in the mixture that is important property for *in-situ* bioremediation.
- Undefined consortium has also shown significant results for cometabolism of 2,4-DCP, but not able to utilize 2,4,6-TCP during cometabolism. However, all other chlorophenols in the mixture with 2,4,6-TCP has shown degradation. In case of 2,4-DCP cometabolism, the presence of MCPs leads to higher total chlorophenols removal rate. The presence of 3-CP and 4-CP contributes to higher degradation rate of 2,4-DCP and also total chlorophenols removal rate as compared to 2-CP which is totally opposite observation than the defined mixed consortium. The biodegradation (mg/L/d) of 2,4-DCP and MCPs takes place in the order of **3CP>4CP>DCP>2CP**.

Comparison of kinetic parameters for degradation of monochlorophenols by both mixed cultures.

Mixed consortium	2-CP			3-CP			4-CP		
	R_m	K_s	K_i	R_m	K_s	K_i	R_m	K_s	K_i
Defined	2.78	956	1061.6	0.91	46.57	189.46	1.82	225.68	323.2
Undefined	-	-	-	1.96	47.23	104.12	6.33	1262	1408.8

R_m = maximum removal rate (mg/L/h); *Ks* = haft saturation constant (mg/L); K_i = substrate inhibition constant (mg/L)

4.7. Biodegradation of 2,4-dichlorophenol in packed bed biofilm reactor-1 (PBBR-1)

The present study focused mainly on two subjects. First was the fed-batch study, in which the HRT (24 h) and peptone (1g/L) concentration was kept constant while the initial substrate concentration was increased stepwise to check maximum concentration that could be treated by PPBR and also to acclimatize the biomass gradually. Second was the continuous operation, divided into three parts. First, the effect of HRT on biodegradation efficiency was studied by gradually decreasing HRT by keeping the substrate concentration constant. Second, the effect of biogenic substrate, i.e. peptone, was studied by keeping the HRT and substrate concentration constant. Finally the effect of loading rate or initial substrate concentration was examined in which the peptone (0.2 g/L) and HRT (12.5±1 h) were kept constant.

The bioreactor was operated before inoculation for two days to check the abiotic loss by evaporation and adsorption to ceramic balls. The air flow rate was set to 0.1 LPM. The MSM containing 50 mg/L of 2,4-DCP was fed to the bioreactor. The results demonstrated that only 3-5% loss in the reactor was due to evaporation by air flow.

The bioreactor was fed with nutrient broth containing 50 mg/L of 2,4-DCP up to three days for biofilm formation. After biofilm formation, the nutrient broth was drained and biofilm was washed with phosphate buffer (pH 7.2) and then used for biodegradation process.

4.7.1. Fed-batch study

The bioreactor was run in fed-batch mode to check the biodegradation capacity and the maximum concentration of 2,4-DCP that could be removed by *B. endophyticus* CP1R strain. The fed-batch mode was operated for 16 days during which the HRT was set to 24 h. The percentage removal of 2,4-DCP during the fed-batch operation is shown in Figure 4.91. The bioreactor was fed with fresh MSM containing varying concentration of 2,4-DCP (10 to 60 mg/L) regularly. The samples were taken after 24 h operation and analyzed for the residual concentration of 2,4-DCP. The bioreactor showed 96-100% removal of 2,4-DCP up to 60 mg/L concentration with 24 h HRT. The biomass in the effluent initially decreased for 40 mg/L 2,4-DCP and then started increasing for 50 mg/L 2,4-DCP. The biomass concentration was observed to decrease for 60 mg/L 2,4-DCP due to increased toxicity and decreased biomass growth.

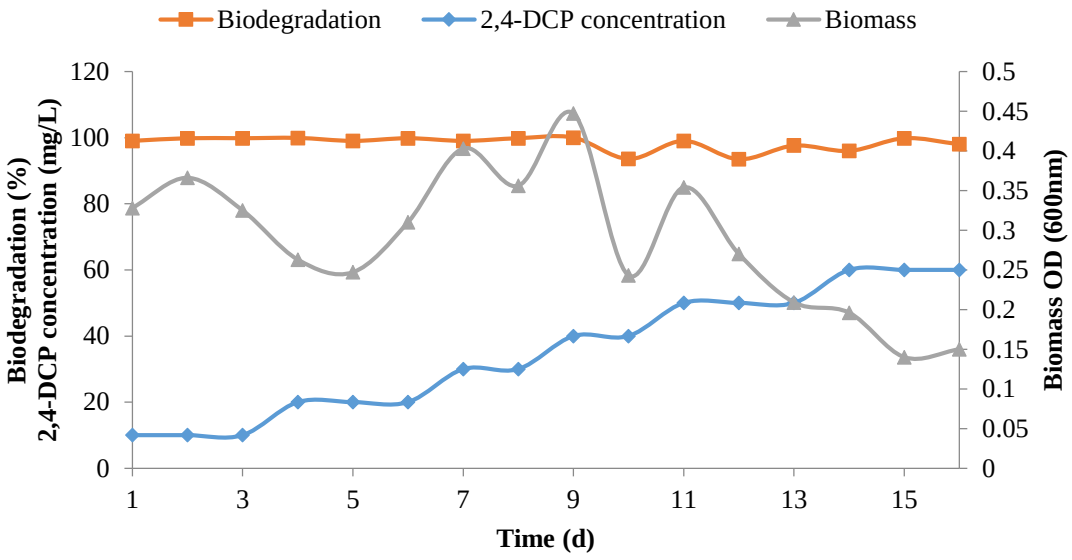


Figure 4.91: Biodegradation of 2,4-DCP by *Bacillus endophyticus* 1R during fed-batch study.

4.7.2. Continuous study

The packed bed biofilm reactor showed excellent removal of 2,4-DCP in continuous mode. The bioreactor was operated for 50 days in a continuous mode during which effect of HRT, initial substrate concentration and peptone concentration on biodegradation of 2,4-DCP by *B. endophyticus* CP1R strain was studied.

4.7.2.1. Effect of HRT

The effect of HRT on the removal of 2,4-DCP was analyzed by keeping the substrate and peptone concentration constant at 60 mg/L and 1 g/L respectively. The HRT was gradually decreased from 30 h to 12.5±1 h. The results showing the effect of HRT on biodegradation of 2,4-DCP and biomass concentration are presented in figure 4.92. All the values shown in the figure are an average taken after bioreactor had achieved equilibrium condition. No drastic change in removal efficiency was observed with decreasing HRT. The bioreactor has shown more than 98% removal of 2,4-DCP at 18.75±1 h HRT and the volumetric removal rate obtained at this condition was 75.26 mg/L/d. There was a decrease in removal efficiency observed when HRT was further decreased to 12.5 h. At equilibrium condition, the bioreactor achieved 94% removal of 2,4-DCP at 12.5±1 h HRT and the volumetric removal rate obtained at this condition was 108.3 mg/L/d. The effect of HRT on the volumetric removal rate of 2,4-DCP is shown in figure 4.93. The volumetric removal rate increases with decreasing HRT and highest removal rate obtained in this study was at 12.5 h HRT. The bioreactor should be operated at HRT of 18.75±1 h or more to achieve greater than 98% removal, and HRT should be kept at 12.5 h to obtain high volumetric removal rate.

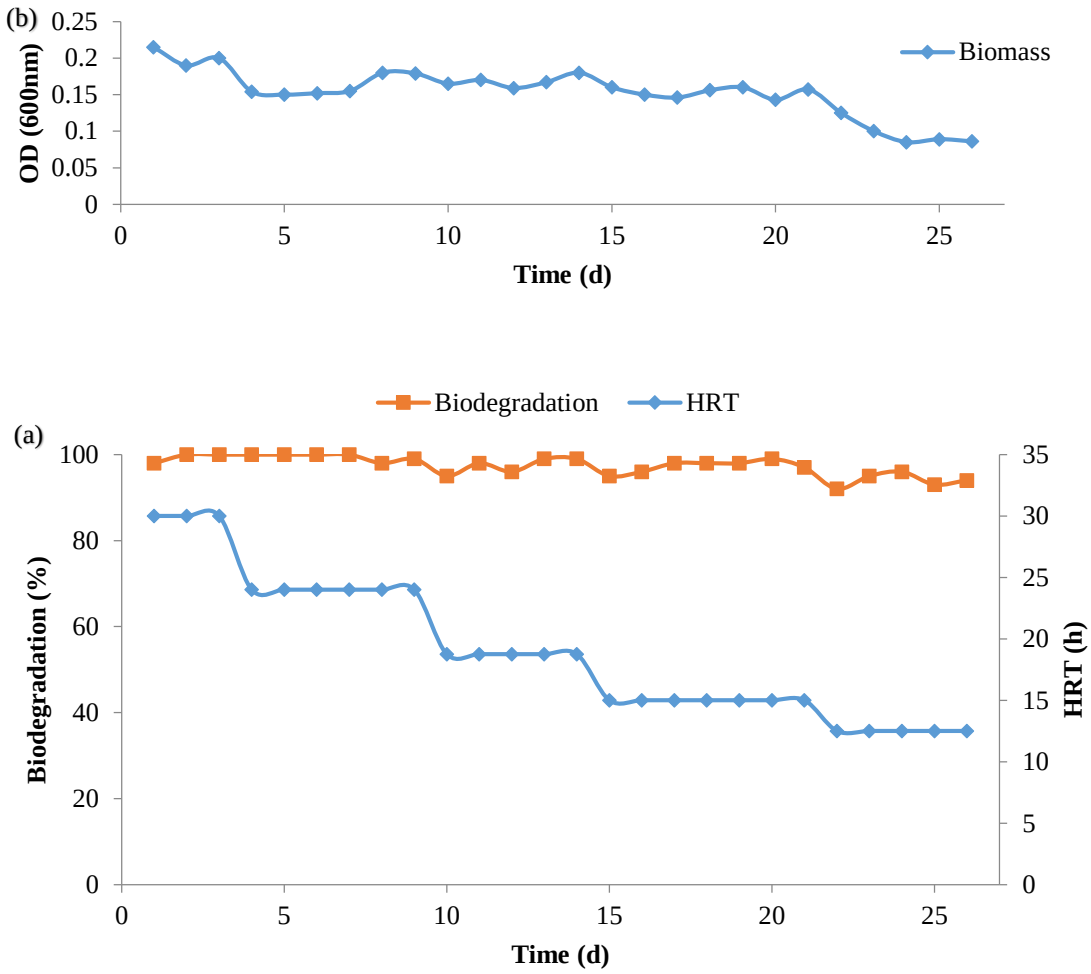


Figure 4.92: (a) Effect of hydraulic retention time on continuous biodegradation of 2,4-DCP (60 mg/L) by *B. Endophyticus* 1R in PBBR in the presence of 1g/l peptone. (b) Variation of biomass concentration in the effluent throughout the operation.

Table 4.24: Effect of HRT on biodegradation of 2,4-DCP by *B. endophyticus* CP1R in PBBR

Time (d)	IC	HRT ±1 (h)	Loading rate (mg/L/d)	Biodegradation (%)	Volumetric removal rate (mg/L/d)
1-3	60	30	48	100	48
4-9	60	24	60	99.4	59.64
10-14	60	18.75	76.8	98	75.26
15-21	60	15	96	97.5	93.6
22-26	60	12.5	115.2	94	108.28

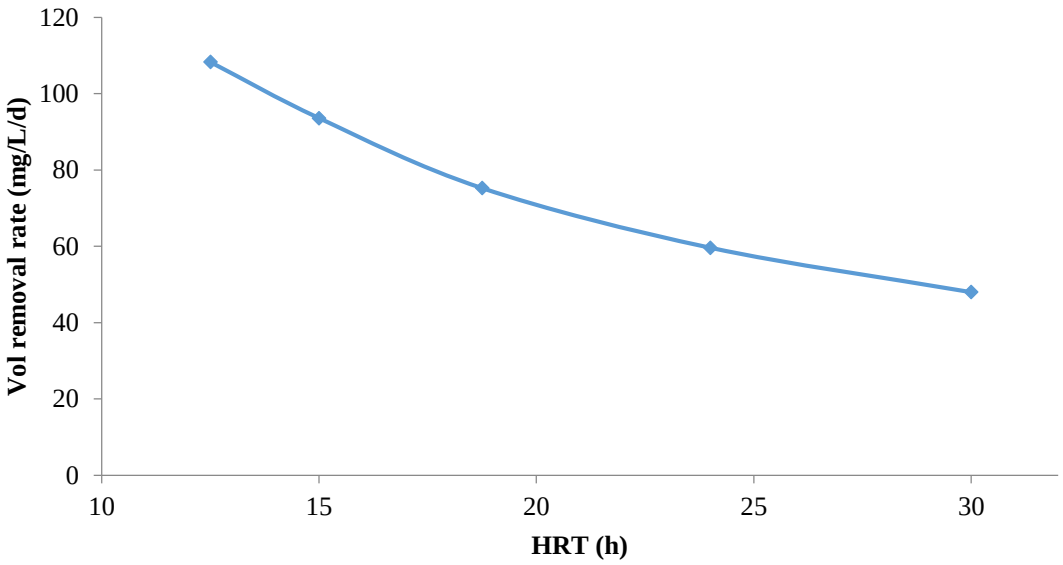


Figure 4.93: Effect of HRT on the volumetric removal rate of 2,4-DCP (60 mg/L) in the PBBR

4.7.2.2. Effect of Peptone

Effect of peptone as biogenic substrate was studied by keeping substrate concentration constant at 60 mg/L and gradually decreasing HRT from 18.75 to 12.5±1 h. Peptone concentration was decreased from initial 1g/L to 0.2 g/L. A previous study has shown that 0.2 g/L peptone is required to boost the growth of the isolate. The bioreactor performance was not much affected when the peptone concentration decreased from the 1 g/L to 0.2 g/L. However, the removal efficiency was observed to increase slightly at 0.2 g/L peptone showing that the microorganism was able to utilize 2,4-DCP as a carbon and energy source. Also, the cost of the process decreases due to less utilization of peptone making the process more economical. At 12.5 h HRT and 60 mg/L initial 2,4-DCP concentration, the bioreactor showed 98% and 94 % removal in the presence of 0.2 and 1 g/L peptone respectively. The biomass concentration remains almost constant in both the conditions.

The peptone may serve as a nitrogen source for the isolate as it had been used during the acclimatization period. Therefore, presence of peptone at low concentration is important to obtain maximum degradation. Also, it was observed that the biomass concentration remains almost constant on reducing the peptone concentration in the medium inferring that the peptone did not contribute to biomass growth leading to the conclusion that the isolate utilized the 2,4-DCP as carbon and energy source only.

4.7.2.3. Effect of initial substrate concentration

Effect of initial substrate concentration on bioreactor performance was studied by keeping the HRT (12.5±1 h) and peptone (0.2 g/L) concentration constant and gradually

increasing the substrate concentration. When the substrate concentration was increased from 60 to 90 mg/L at constant HRT, biodegradation efficiency decreased from 98% to 73.6%. The biomass concentration in the effluent was also decreased with substrate concentration as shown in figure 4.94. However, volumetric removal rate (mg/L/d) increased with loading rate up to 134.4 mg/L/d and remained almost constant up to 172.8 mg/L/d as shown in figure 4.95. The highest volumetric removal rate observed was 127.2 mg/L/d at a loading rate of 172.8 mg/L/d with 0.2 g/L peptone. Loading rate should be kept below 115.2 mg/L/d to achieve more than 98% removal of 2,4-DCP in the bioreactor. However to obtain maximum removal rate, loading rate should be kept at 172.8 mg/L/d. Effect of loading rate on the removal rate of 2,4-DCP at constant HRT has been presented in figure 4.95.

At lower concentrations of 2,4-DCP, no intermediate was detected by HPLC analysis. However at higher concentration, the presence of trace amount of intermediate metabolites was observed. Mass spectroscopy analysis of the effluent revealed the presence of single chlorinated and non-chlorinated compounds indicating the degradation of 2,4-DCP. The pH value of effluent was observed to decrease to 7.0±1 compare to influent pH value of 7.35. This drop in pH value was due to the release of chloride ion indicating the biodegradation of 2,4-DCP.

Table 4.25. Effect of peptone and loading rate on biodegradation of 2,4-DCP by *B. endophyticus* CP1R in PBBR

Time (d)	IC	HRT ±1 (h)	Loading rate (mg/L/d)	Biodegradation (%)	Volumetric removal rate (mg/L/d)
29-32	60	18.75	76.8	94.8	72.8
33-36	60	15	96	98.6	94.65
37-39	60	12.5	115.2	98	112.89
40-43	70	12.5	134.4	95	127.68
44-47	80	12.5	153.6	83.4	128.1
48-50	90	12.5	172.8	73.6	127.18

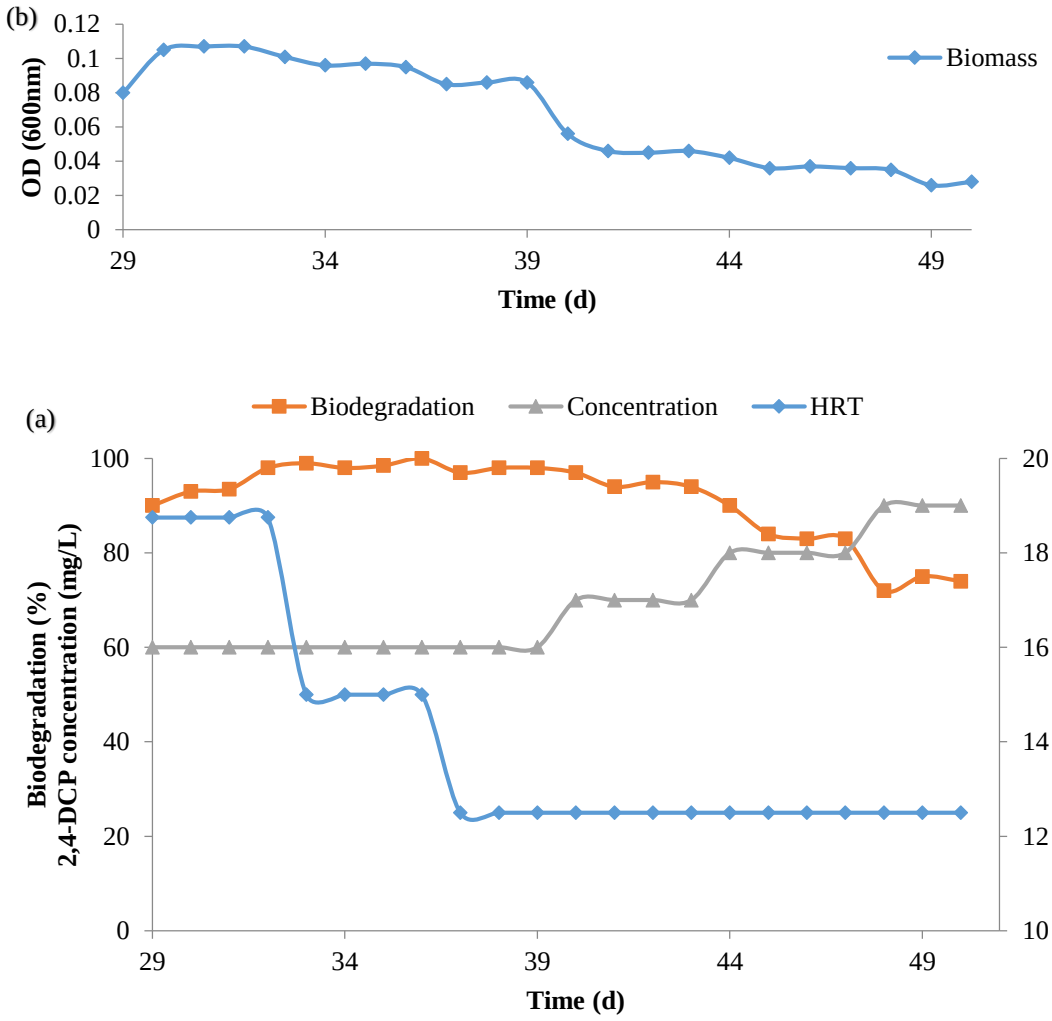


Figure 4.94: (a) Effect of peptone and initial substrate concentration on the removal of 2,4-DCP in PPBR by *B. Endophyticus* 1R in the presence of 0.2 g/L of peptone. (b) Variation of biomass concentration in the effluent throughout the operation.

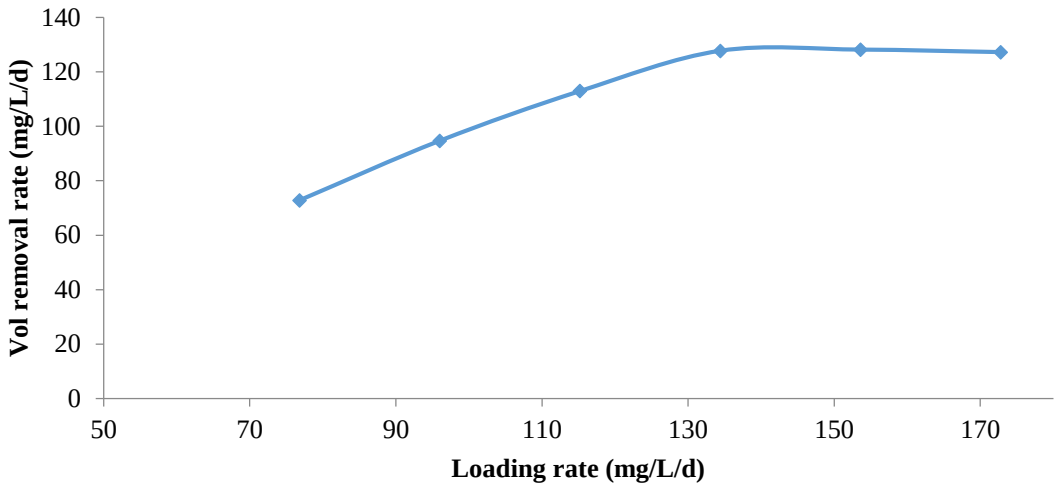


Figure 4.95: Effect of loading rate on the volumetric removal rate of 2,4-DCP in the PBBR.

4.7.3. Biofilm formation

FESEM analysis confirmed biofilm formation. For FESEM investigation, ceramic beads were taken from the top of the bioreactor during at 34th day of the operation. The beads were treated with 2% glutaraldehyde for 2 h for cell fixation. After fixation, the beads were washed/dehydrated with series of ethanol (30%, 50%, 70% and 90%) for 10 min at each concentration. Afterward, the beads were washed three times with 100% ethanol for 10 min each. After drying, the beads were analyzed by FESEM. The attached biofilm can be visualized on the ceramic ball as shown in figure 4.96. The microorganism has rod shape which is characteristic of the most *Bacillus* species. Also, the microorganisms were observed to form biofilm with an extracellular matrix.

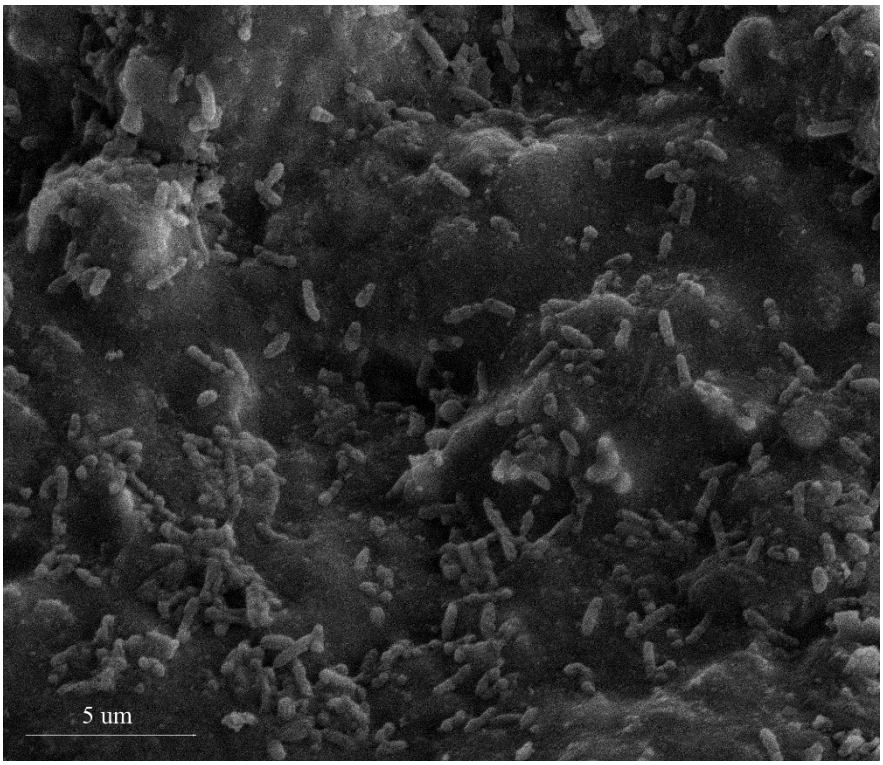
Results demonstrate that Bacillus endophyticus strain 1R can effectively bioremediate 2,4-dichlorphenol in wastewater. The PBBR is able to remove the chlorophenols present in the wastewater before releasing it into the mainstream water. The bioreactor showed more than 98% removal of 2,4-DCP in the effluent for loading rate of 115 mg/L/d. The ceramic beads have also shown good potential for attachment of microorganisms thus forming a suitable support material for biofilm formation. Table 4.26 summarizes the removal of chlorophenols in the different bioreactor systems.

Most of the chlorophenols biodegradation studies are co-metabolic in nature. There are several reports on the biodegradation of chlorophenols in the presence of other carbon and nitrogen source such as glucose, dextrose, peptone, yeast extract, etc. Also, the cometabolism of higher chlorophenol in the presence of lower chlorophenol and phenol has been reported. The presence of secondary carbon and nitrogen source or lower phenols witnessed an increase in the degradation of chlorophenols by contributing the biomass growth. However, there are others reports that publicized the opposing outcomes that sometimes the degradation of higher

chlorophenols were inhibited. The present study reveals that the peptone concentration has not much effect on the degradation of 2,4-DCP. However at low peptone concentration, the removal efficiency increased but some peptone was found to be necessary to maintain the removal efficiency. Sahinkaya and Dilek (2006) studied the effect of peptone on the degradation of 4-CP in a sequencing batch reactor using a mixed consortium. They also reported that as peptone concentration decreases, the specific degradation rate of 4-CP increases [16]. Shen et al. (2005) also described the beneficial nature of the presence of a suitable quantity of microbial easily degradable substrate for stimulating the process of dechlorination and degradation of chlorophenols [92].

Dilaver and kargi, (2009) reported the removal of 2,4-DCP, COD, and toxicity at a different initial substrate concentration in a hybrid loop bioreactor consisting of packed column biofilm reactor and an aerated tank with recirculation. They have shown that initial 2,4-DCP should be kept below 100 mg/L to obtain removal of 90% at constant HRT and SRT (sludge retention time) [231]. Quan et al. (2003, 2004) reported the use of ceramic honeycomb filter for immobilization of microbes in the airlift reactor. They have shown that the presence of phenol inhibited the degradation of 2,4-DCP. Immobilization of microbes on the ceramic filter has contributed to higher removal efficiency of 2,4-DCP by maintaining the microbes in the reactor [21, 100]. Effect of substrate concentration and loading rate on the removal rate of 2,4-DCP in rotating biological contactor (RBC) was demonstrated[232]. This study had shown that removal rate decreased from 99.5 to 92.2% when loading rate increased from 0.36 $g/m^2/d$ to 6.15 $g/m^2/d$.

(a)



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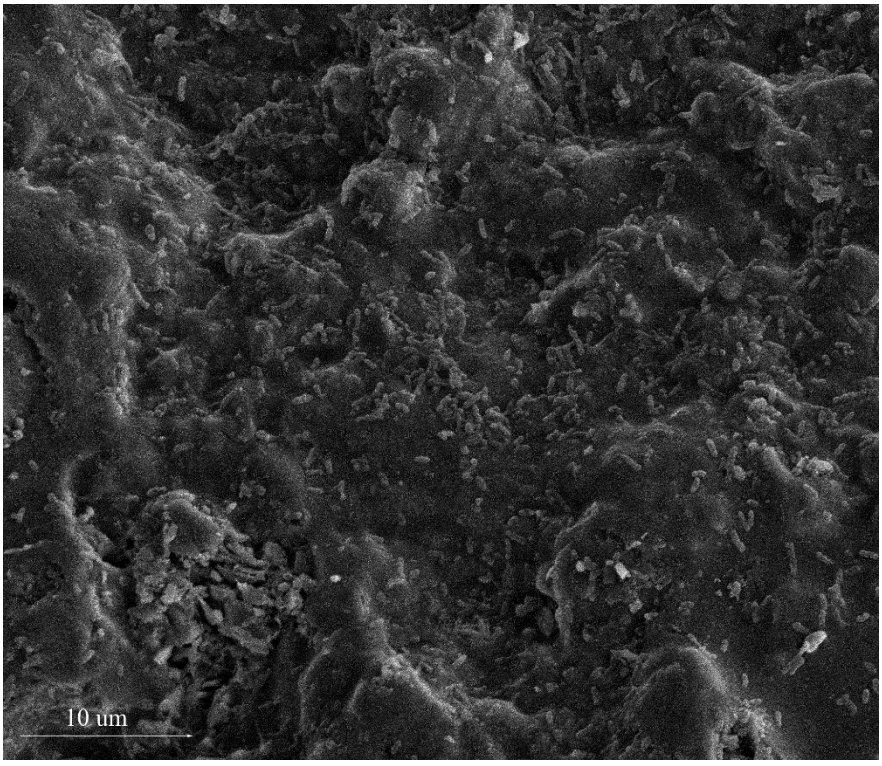

Figure 4.96: TheFE-SEM image of biofilm formation of *B. endophyticus* CP1R in PBBR during biodegradation of 2,4-DCP. (a) at 5000x (b) at 10000x.

Table 4.26: Biodegradation of chlorophenols under different bioreactor system

Reactor	Compounds	Microorganism	HRT (h)	Loading rate (mg/L/d)	Removal rate (%)	Reference
ALR	2,4-DCP Phenol	*Achromobacter* sp.	8	85.5	97.8	[100]
UASB	2,4-DCP Glucose	Mixed culture	13.2	226	70.4	[23]
ALR	2,4-DCP	*Achromobacter* sp.	6.25	26 – 394	100-88	[100]
PBR	4-CP Phenol	*P. testosteroni* CPW301	2.78	172	100	[21]
PCBR	2,4-DCP	Activated sludge	20	456	55	[139]

	Sucrose					
ASB	2,4-DCP	Activated sludge	17	141	98.6	[233]
	Glucose					
PBBR	2,4-DCP	*B. endophyticus* CP1R	12.5	172.8	73.6	This study
	peptone					

PCBR (Packed column biofilm reactor with aeration tank); PBR (Packed bed reactor); ALR (Airlift reactor); UASB (Upflow anaerobic sludge blanket); ASB (Activated sludge bioreactor)

4.8. Removal of 4-chlorophenol by the defined mixed consortium in PBBR-2

The present study focused on continuous removal of 4-CP by the defined mixed consortium in packed bed biofilm reactor which was divided into three parts. First, the effect of HRT on the removal efficiency of bioreactor was studied by gradually decreasing the HRT while keeping substrate concentration constant. Second, the effect of peptone as a biogenic substrate on the biodegradation efficiency was evaluated. Finally the effect of initial substrate concentration at constant HRT or loading rate on biodegradation of 4-CP was studied.

The bioreactor was operated for two days before inoculation to check the abiotic loss of 4-CP. The bioreactor was fed with MSM containing 40 mg/L of 4-CP. The result showed negligible abiotic loss of 4-CP (data not shown).

For biofilm formation, the bioreactor was filled with nutrient broth containing 20 mg/L of 4-CP and inoculated with 10% (v/v) defined mixed microbial consortium. The inoculum was prepared in MSM containing 25 mg/L of 4-CP and cultured for 24 h. The bioreactor was operated for three days and the biofilm formation was confirmed by visually and microscopically. The nutrient broth was drained, and the biofilm was washed with phosphate buffer (pH 7.2) and after that the biodegradation process was started. The biodegradation experiment was performed using MSM prepared from filtered tap water.

The bioreactor was operated for 30 days in continuous mode for removal of 4-CP during which the effect of HRT, loading rate and peptone concentration was evaluated. The bioreactor showed great performance for removal of 4-CP as sole carbon and energy source in continuous mode.

4.8.1. Effect of HRT

The effect of HRT on the removal of 4-CP was studied by keeping the initial substrate concentration (40 mg/L) and peptone concentration (1 g/L) constant and gradually decreasing the HRT. The initial substrate concentration selected was 40 mg/L based on the result obtained in previous batch studies. The peptone concentration was kept 1g/L, as the 4-CP is more toxic and inhibits the microbial growth. So to increase the biomass growth and biofilm formation during the startup of the process, peptone concentration was initially kept high. The HRT was set 30 h during the startup of the process. The HRT was controlled by the changing the flow rate. The effect of HRT on the removal of 4-CP by the defined mixed consortium in PBBR is shown in figure 4.97. The bioreactor achieved 100% removal of 4-CP at 30 h HRT at steady state. There was no drastic change in removal efficiency observed when HRT was decreased to 24 h. The removal efficiency decreased to 96% at steady state for 24 h HRT. Further decrease in HRT to 19.5 h, the biodegradation of 4-CP decreased to 85% at equilibrium. The biomass concentration was not much changed during this period as shown in figure 4.97. The biomass concentration in the effluent was observed to remain steady for HRT between 24 and 19.5 h. However, when HRT was decreased to 15 h, the biomass growth was inhibited, and a washout occurred leading

to diminished biomass growth in the bioreactor medium. The biodegradation efficiency at 15 h HRT was observed to be 66%.

Figure 4.98 shows the effect of HRT on the volumetric removal rate and biodegradation of 4-CP in PBBR. As shown in the figure, the volumetric removal rate increases with decreasing HRT. However, the biodegradation rate was higher at 30 h HRT and decreased to 66% at 15 h HRT. At 30 h HRT, the removal rate observed was 1.34 mg/L/h which increased to 1.76 mg/L/h at 15 h HRT. After 24 h HRT, there was no drastic increase in removal rate observed but it remained almost steady. So, for maximum volumetric removal, the HRT should be kept at 15 h, and for achieving maximum biodegradation of 4-CP, the HRT should be kept at 30 h.

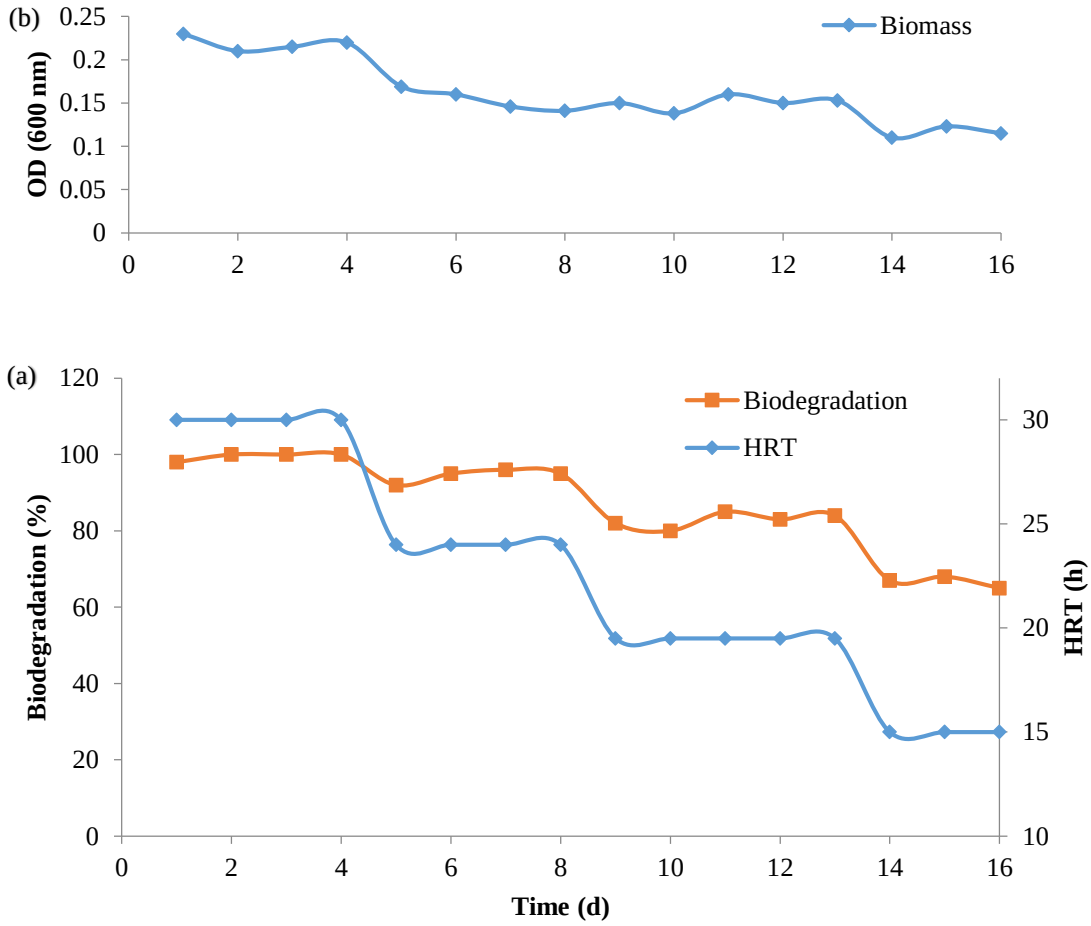

Figure 4.97: (a) The effect of HRT on the continuous removal of 4-CP in the presence of 1g/L peptone by defined mixed microbial consortium in PBBR. (b) Variation of biomass concentration in the effluent throughout the operation.

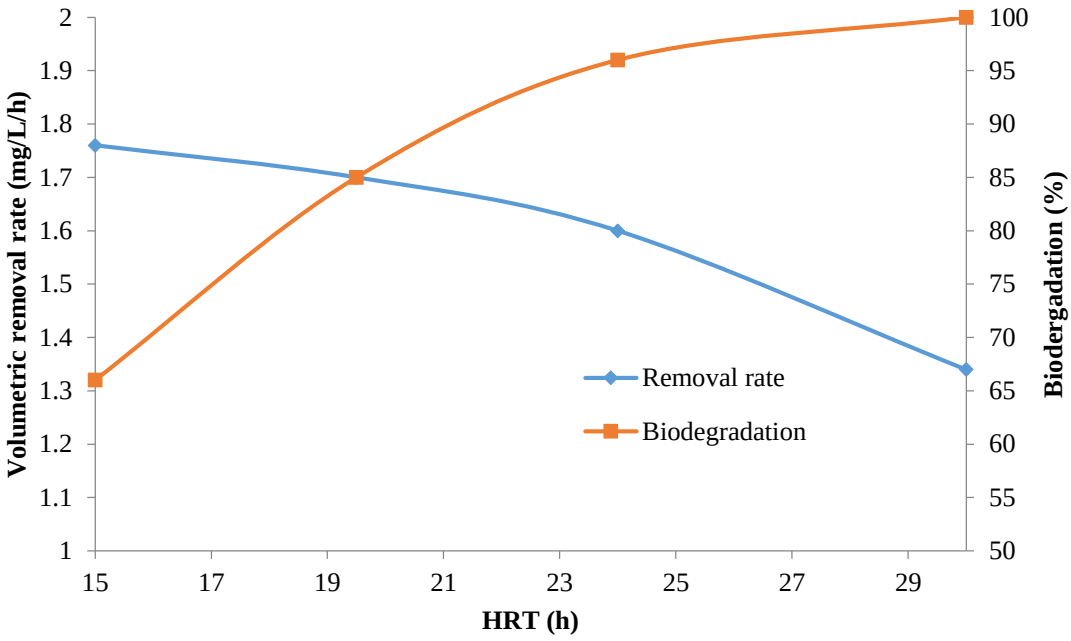

Figure 4.98: The effect of HRT on volumetric removal rate and biodegradation of 4-CP in the presence of 1 g/L of peptone by the defined mixed consortium in PBBR.

4.8.2. Effect of peptone concentration

The effect of peptone concentration on the removal of 4-CP was evaluated by keeping initial 4-CP concentration (40 mg/L) constant. The peptone concentration was decreased from 1 g/L to 0.2 g/L at 15 h HRT. The biodegradation was decreased from 66% to 60% at steady state. There was no drastic change in biodegradation observed when peptone concentration decreased five times to initial concentration. To check the effect of HRT on this condition (0.2 g/L peptone), the HRT was gradually increased from 15 h to 30 h. It was observed that the biodegradation of 4-CP gradually increased from 60% to 94% at steady state. The effect of peptone and HRT on 4-CP removal is shown figure 4.99. Also at this condition, the biomass growth was increased in the medium as shown in figure 4.99. The air flow was increased from 0.15 LPM to 2 LPM on the 18th day of the continuous operation. This increase in air flow leads to increase in dissolved oxygen. Also, it had a positive effect on biomass growth and biodegradation. The bioreactor has achieved 94% biodegradation of 40 mg/L of 4-CP at 30 h HRT in the presence of 0.2 g/L of peptone which was slightly less than that obtained in the presence of 1 g/L of peptone i.e. 100%. The results showed that the biodegradation of 4-CP in PBBR was not much affected by the change in peptone concentration. Also, the mixed consortium was able to utilize the 4-CP as a carbon and energy source as evidenced by an increase in biomass growth at low peptone concentration.

4.8.3. Effect of initial substrate concentration

The effect of initial 4-CP concentration or loading rate on removal efficiency was studied by the keeping HRT (30 h) and peptone concentration (0.2 g/L) constant. The initial 4-CP concentration in influent was gradually increased from 40 mg/L to 90 mg/L. The biodegradation rate decreased from 94% at 40 mg/L to 73% at 90 mg/L of initial substrate concentration (Figure 4.99). There was no apparent change in biomass growth observed at this condition. Figure 4.100 shows the effect of loading rate on the volumetric removal rate of 4-CP in the presence of 0.2 g/L and 1 g/L of peptone. At 1g/L peptone concentration, there was no apparent change in removal rate was observed after 40 mg/L/d of loading rate. While at 0.2 g/L of peptone concentration, the removal rate was increased almost linearly with loading rate. Also, the volumetric removal rate was found to be higher for 0.2 g/L of peptone than that obtained in the presence of 1 g/L of peptone (Figure 4.100). The maximum removal rate obtained was 2.14 mg/L/h at a loading rate of 63 mg/L/d in the presence of 0.2 g/L of peptone.

4.8.4. Biofilm formation

FESEM analysis confirmed biofilm formation. For FESEM investigation, ceramic beads were taken from the top of the bioreactor during at 25th day of the operation. The beads were preapared for FESEM analysis as per method described in section 4.7.3. The attached biofilm can be visualized on the ceramic ball as shown in figure 4.101. The microorganisms were mostly rod-shaped, and some of them are small round shaped. Also, the microorganisms are observed to be form biofilm with an extracellular matrix inside the micropores of the ceramic beads.

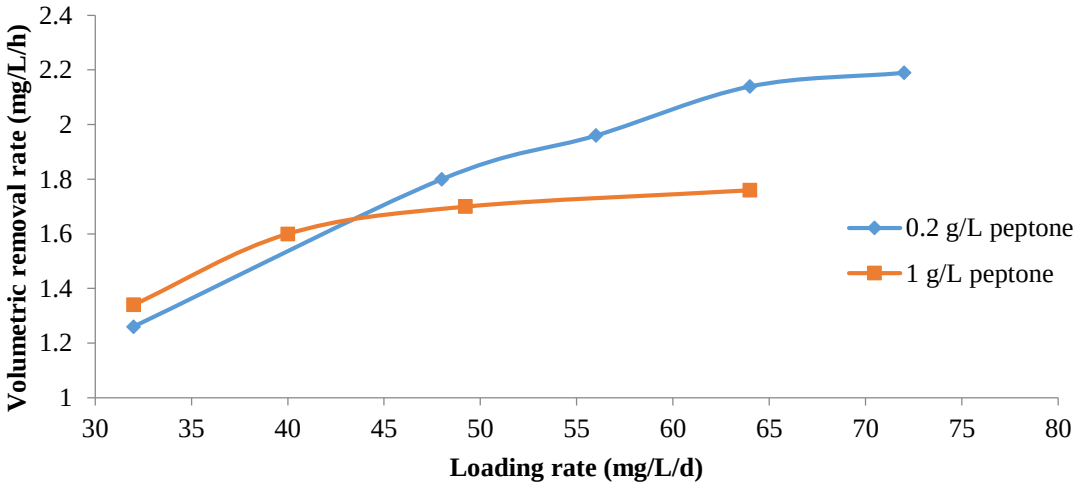

Figure 4.100: The effect of loading rate on the volumetric removal rate of 4-CP by the defined mixed consortium in the presence of 0.2 and 1 g/L of peptone in PBBR.

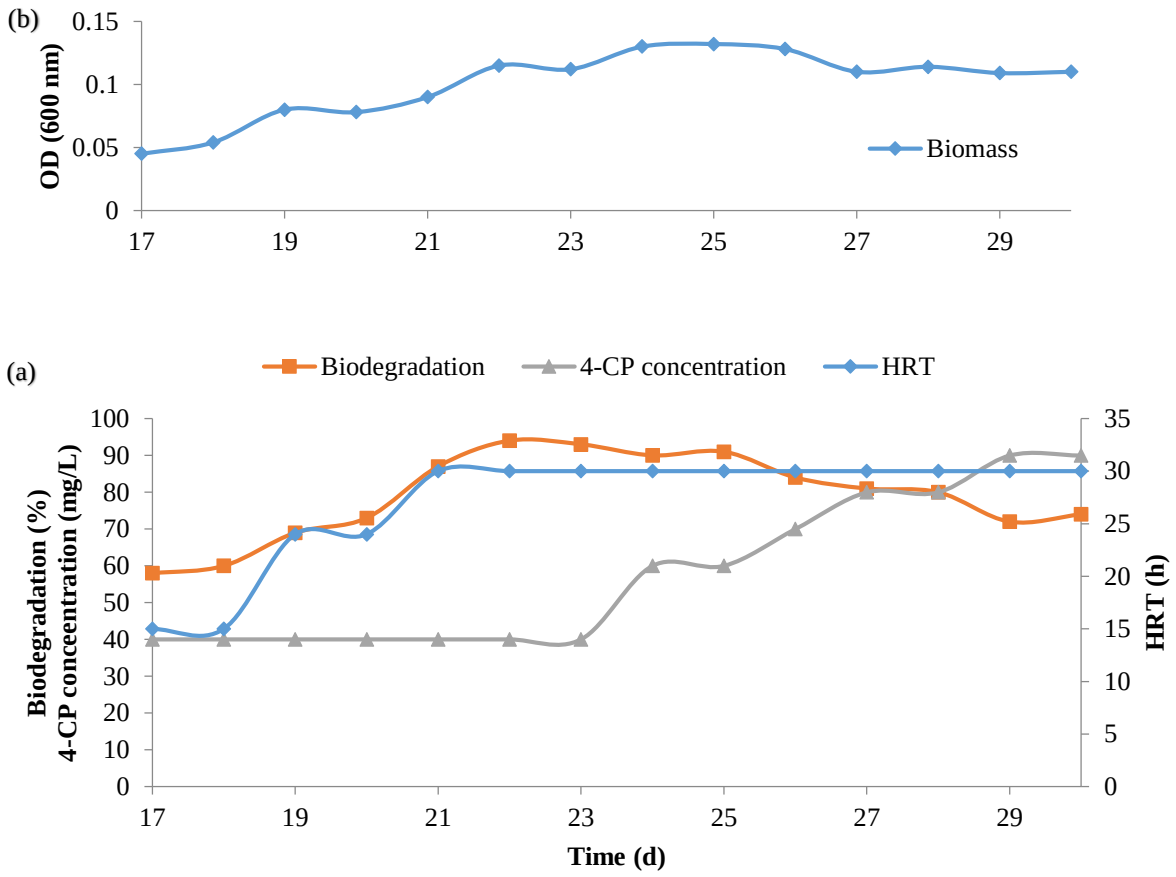

Figure 4.99: (a) The effect of initial 4-CP concentration and peptone concentration on the continuous removal of 4-CP by defined mixed microbial consortium in the presence of 0.2 g/L peptone in PBBR. (b) Variation of biomass concentration in the effluent throughout the operation.

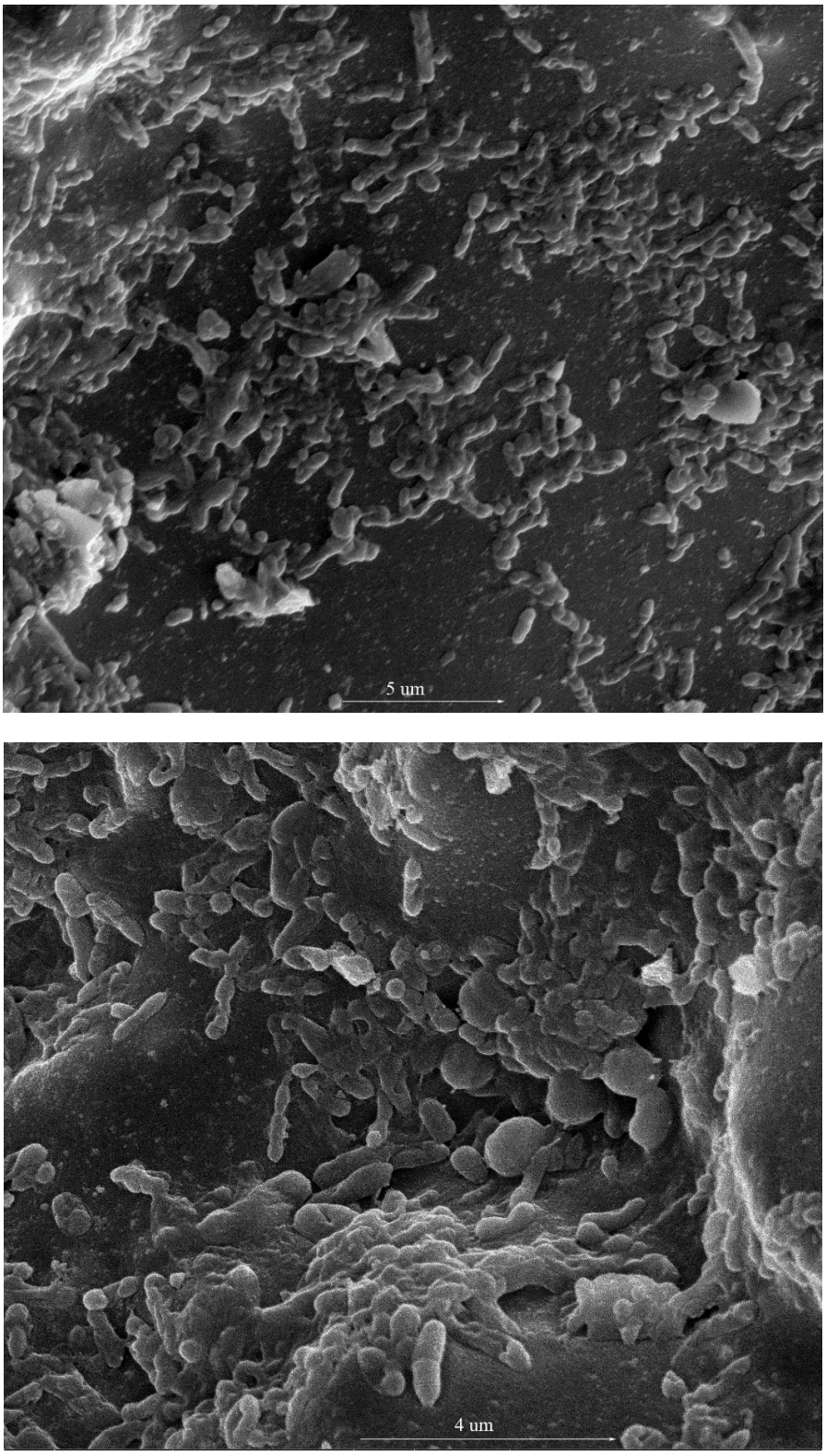

Figure 4.101: FESEM image of biofilm formation during the biodegradation of 4-CP by the defined mixed consortium in PBBR-2.

4.9. Removal of 3-chlorophenol by the defined mixed consortium in PBBR-2

The present study focused on continuous removal of 3-CP by the defined mixed consortium in packed bed biofilm reactor that was divided into three parts. First, the effect of HRT on the removal efficiency of bioreactor was studied by gradually decreasing the HRT while keeping substrate concentration constant. Second, the effect of initial substrate concentration at constant HRT or effect of loading rate on biodegradation of 3-CP was studied. Finally, the effect of peptone as a biogenic substrate on the biodegradation efficiency was evaluated.

The bioreactor was operated for two days before inoculation to check the abiotic loss of 3-CP. The bioreactor was fed with MSM containing 40 mg/L of 3-CP, and the result showed negligible abiotic loss of 3-CP in the bioreactor.

For biofilm formation, the bioreactor was filled with nutrient broth containing 20 mg/L of 3-CP and inoculated with 10% (v/v) mixed microbial consortium. The inoculum was prepared in MSM containing 25 mg/L of 3-CP and cultured for 24 h. The bioreactor was operated for three days and the biofilm formation was confirmed by visually and microscopically. The nutrient broth was drained, and the biofilm was washed with phosphate buffer (pH 7.2) and after that the biodegradation process was started. The biodegradation experiment was performed using MSM prepared from filtered tap water.

The bioreactor was operated for 35 days in continuous mode for removal of 3-CP during which the effect of HRT, loading rate and peptone concentration was evaluated. The bioreactor showed excellent removal of 3-CP in continuous mode.

4.9.1. Effect of HRT

The effect of HRT on the removal of 3-CP by the defined mixed consortium in PBBR was evaluated by keeping initial 3-CP and peptone concentration constant at 40 mg/L and 0.2 g/L respectively. The HRT was gradually decreased from 30 h to 18 h. The continuous operation was started with 20 mg/L of 3-CP and then increased to 40 mg/L. The bioreactor has shown 99% removal of 3-CP for 20 mg/L of initial substrate concentration at 30 h HRT. The biodegradation rate was not much affected when initial 3-CP concentration was increased to 40 mg/L and became steady at 97% at 30 h HRT. The effect of HRT on biodegradation is shown in figure 4.102. Also, the biomass concentration in the medium increased when substrate concentration increased as shown in figure 4.102. The biodegradation rate was decreased to 82% when HRT was decreased to 24 h. A further change in HRT from 24 to 18 h, the biodegradation rate was reduced drastically to 56% at steady state. The biomass concentration in the medium was also decreased when HRT was decreased from 30 to 18 h due to washout of the cells and inhibition of the cell growth at high loading rate. The previous batch study also shows that the 3-CP is more toxic and has inhibited the microbial growth even at low concentration. The HRT was again increased to 30 h, to check if the bioreactor regains its biodegradation efficiency. The result showed that when the HRT was increased to 30 h, the removal efficiency of the bioreactor

gradually increased and became steady at 97% (Figure 4.102). The microbes were also regained growth and biomass concentration increased in the bioreactor medium. Figure 4.103 shows the effect of HRT on the volumetric removal rate of 3-CP in PBBR. The maximum volumetric removal rate achieved was 1.38 mg/L/h at 24 h HRT.

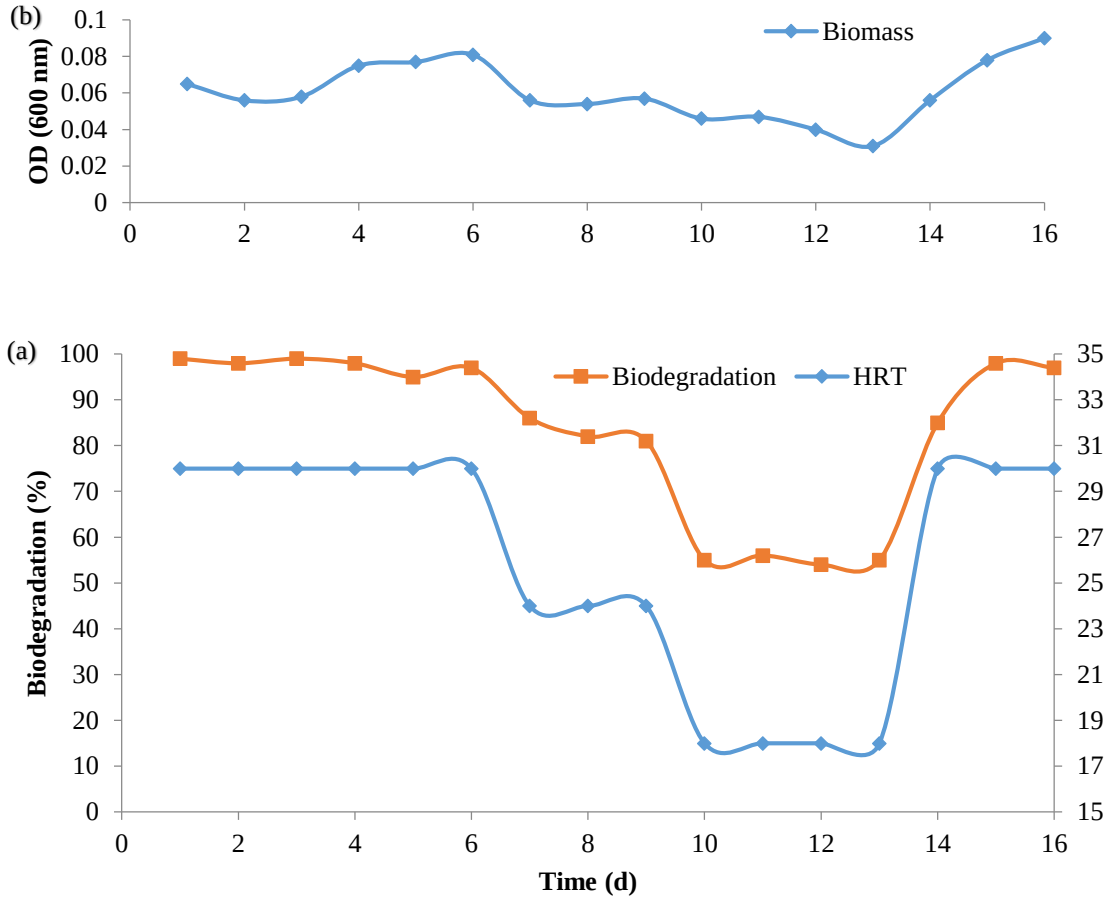

Figure 4.102: (a) The effect of HRT on the continuous removal of 3-CP in the presence of 0.2 g/L peptone by defined mixed microbial consortium in PBBR. (b) Variation of biomass concentration in the effluent throughout the operation.

4.9.2. Effect of Initial 3-CP concentration

The effect of initial 3CP concentration or loading rate on the removal efficiency of the PBBR was evaluated by keeping HRT and peptone concentration constant at 30 h and 0.2 g/L. The initial 3-CP concentration in the influent was increased gradually from 40 to 70 mg/L. The biodegradation rate was decreased gradually from 97% to 57% as shown in figure 4.104. During this period, it was also observed that the biomass concentration remained almost constant as shown in figure 4.104. Figure 4.105 shows the effect of loading rate on volumetric removal rate and biodegradation rate of 3-CP in the presence of 0.2 g/L of peptone. The biodegradation rate was decreased almost linearly with increasing loading rate. However, the volumetric removal

rate was initially increased with loading rate and obtained maximum removal rate of 1.52 mg/L/h at 48 mg/L/d loading rate. Further increase in loading rate to 56 mg/L/d, the removal rate was decreased to 1.33 mg/L/h. The higher concentration of 3-CP is toxic and requires a secondary carbon source for biomass growth. The batch study also shows that the mixed consortium required 30-32 h to degrade 50 mg/L of 3-CP.

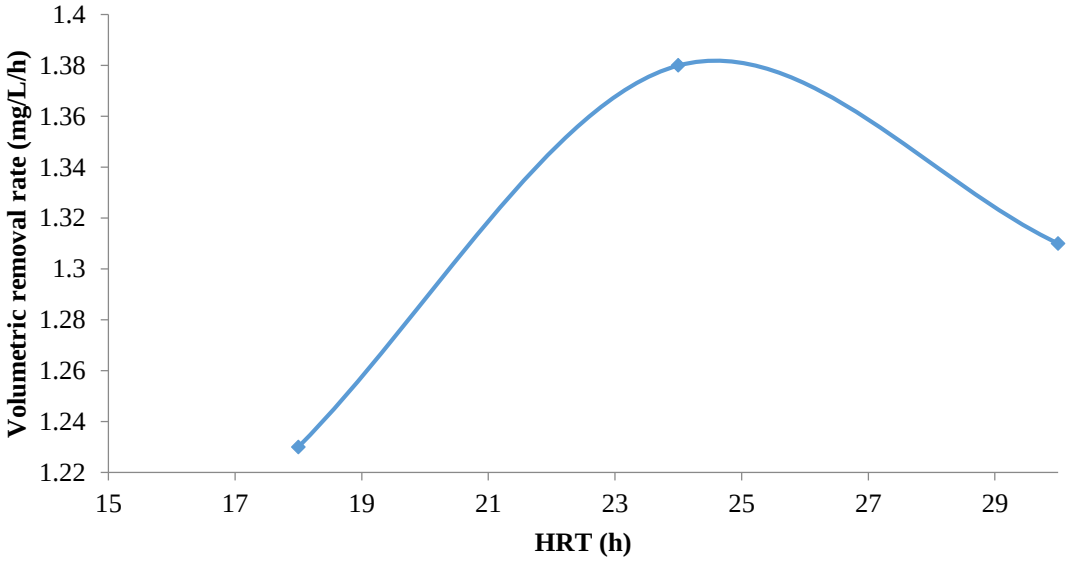

Figure 4.103: Effect of HRT on the volumetric removal rate of 3-CP by the defined mixed consortium in the presence of 0.2 g/L of peptone in PBBR.

4.9.3. Effect of peptone concentration

In the last part of the continuous operation, the effect of peptone concentration on the removal efficiency was studied. The peptone concentration was increased from 0.2 to 1 g/L in the influent on the 25th day of the operation. The HRT and initial 3-CP concentration was kept constant at 30 h and 70 mg/L respectively. It was observed that with increasing peptone concentration, the biodegradation rate of 3-CP was greatly increased and it achieved 96-99% degradation at steady state condition (Figure 4.104). The biomass concentration in the effluent was also greatly increased. In the previous study, it was observed that the removal of 4-CP in PBBR by the defined mixed consortium was not much affected by the peptone concentration. The biodegradation rate observed to increase at low peptone concentration compared to high concentration. But in the case with 3-CP, the peptone has an inverse effect. The biodegradation rate of 3-CP was found to be increased at higher peptone concentration. The 3-CP is more toxic than other two monochlorophenols due to the *meta* positioned chloride ion. The removal of chloride ion from *meta* position is energy demanding process due to electron hindrance. The mixed consortium utilized the peptone as secondary carbon and energy source that leads to increased biomass growth as shown in figure 4.104. The increase in biomass growth helps in the utilization of 3-CP due which the biodegradation rate increases.

There was another report that also showed the presence of secondary carbon source such as peptone increased the biodegradation of chlorophenol compounds [18]. Shen et al. (2005) described the beneficiary nature of the presence of a suitable quantity of microbial easily degradable substrate for stimulating the process of dechlorination and degradation of pentachlorophenol [92].

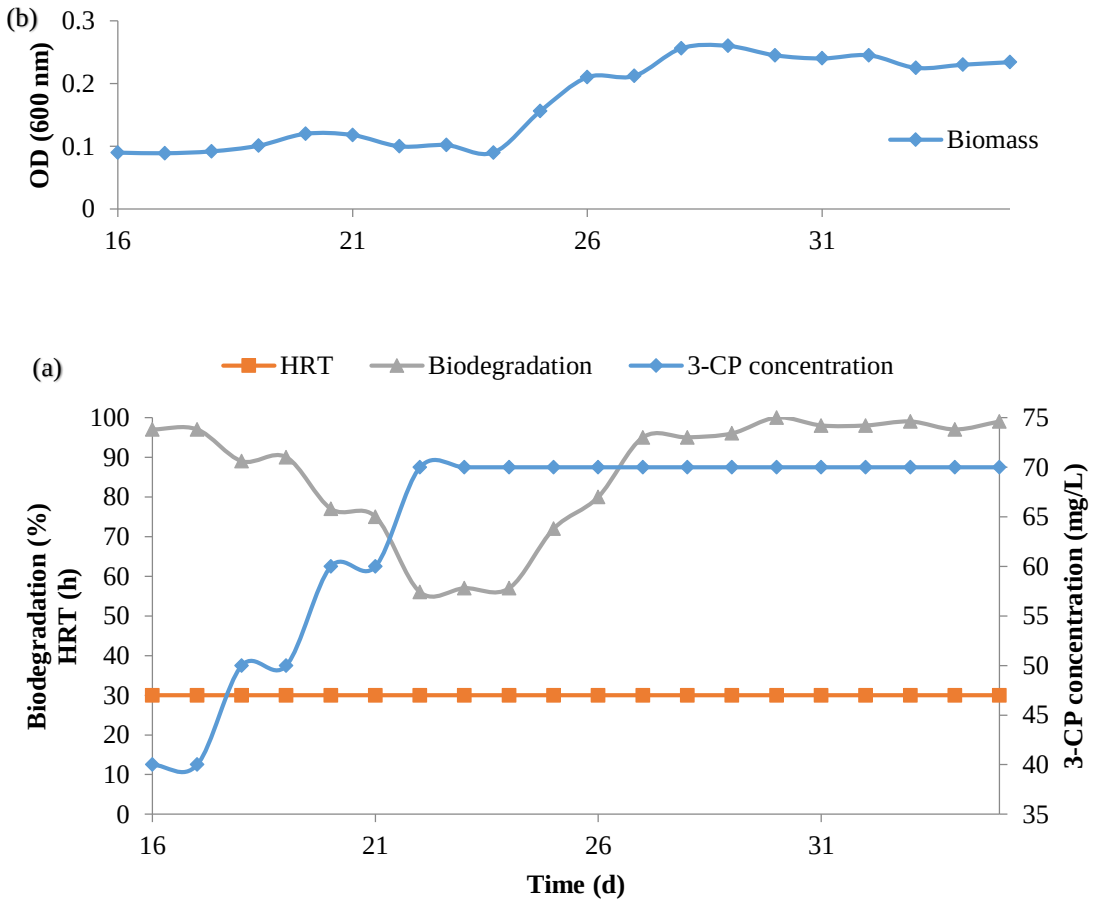

Figure 4.104: (a) The effect of initial 3-CP concentration and peptone concentration on the continuous removal of 3-CP by defined mixed microbial consortium in PBBR. (b) Variation of biomass concentration in the effluent throughout the operation.

4.9.4. Biofilm formation

FESEM analysis confirmed biofilm formation. For FESEM investigation, ceramic beads were taken from the top of the bioreactor during at 20th day of the operation. The beads were preapared for FESEM analysis as per method described in section 4.7.3. The attached biofilm can be visualized on the ceramic ball as shown in figure 4.106. The mixed microorganism can be visualized on the ceramic beads. Most of them has rod shape, and some of them are of the round shape.

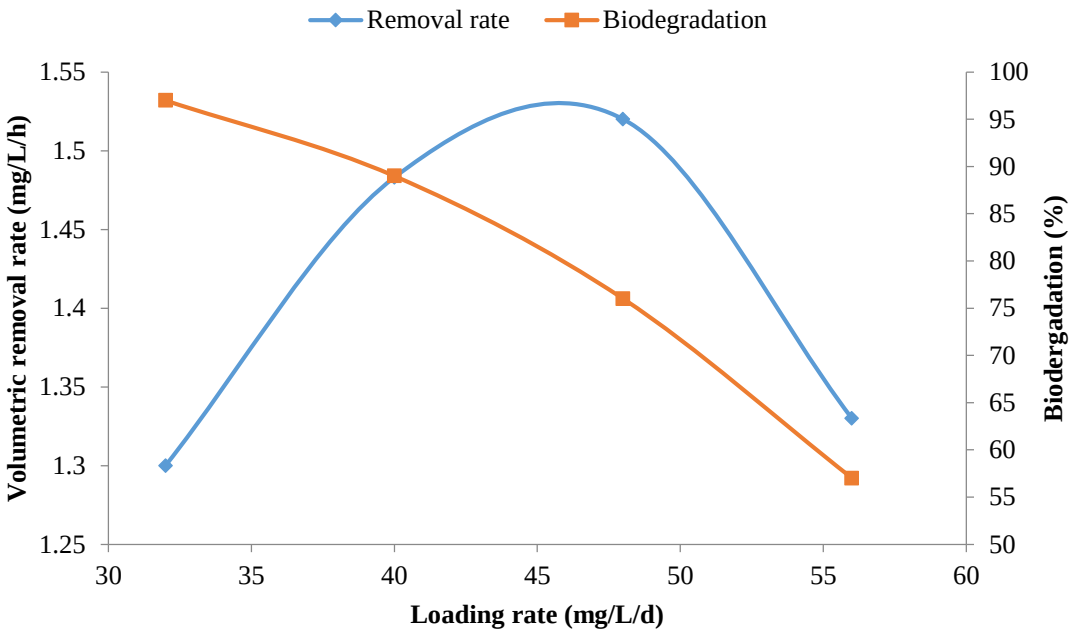

Figure 4.105: The effect of loading rate on volumetric removal rate and biodegradation rate of 3-CP by the defined mixed consortium in the presence of 0.2 g/L of peptone in PBBR.

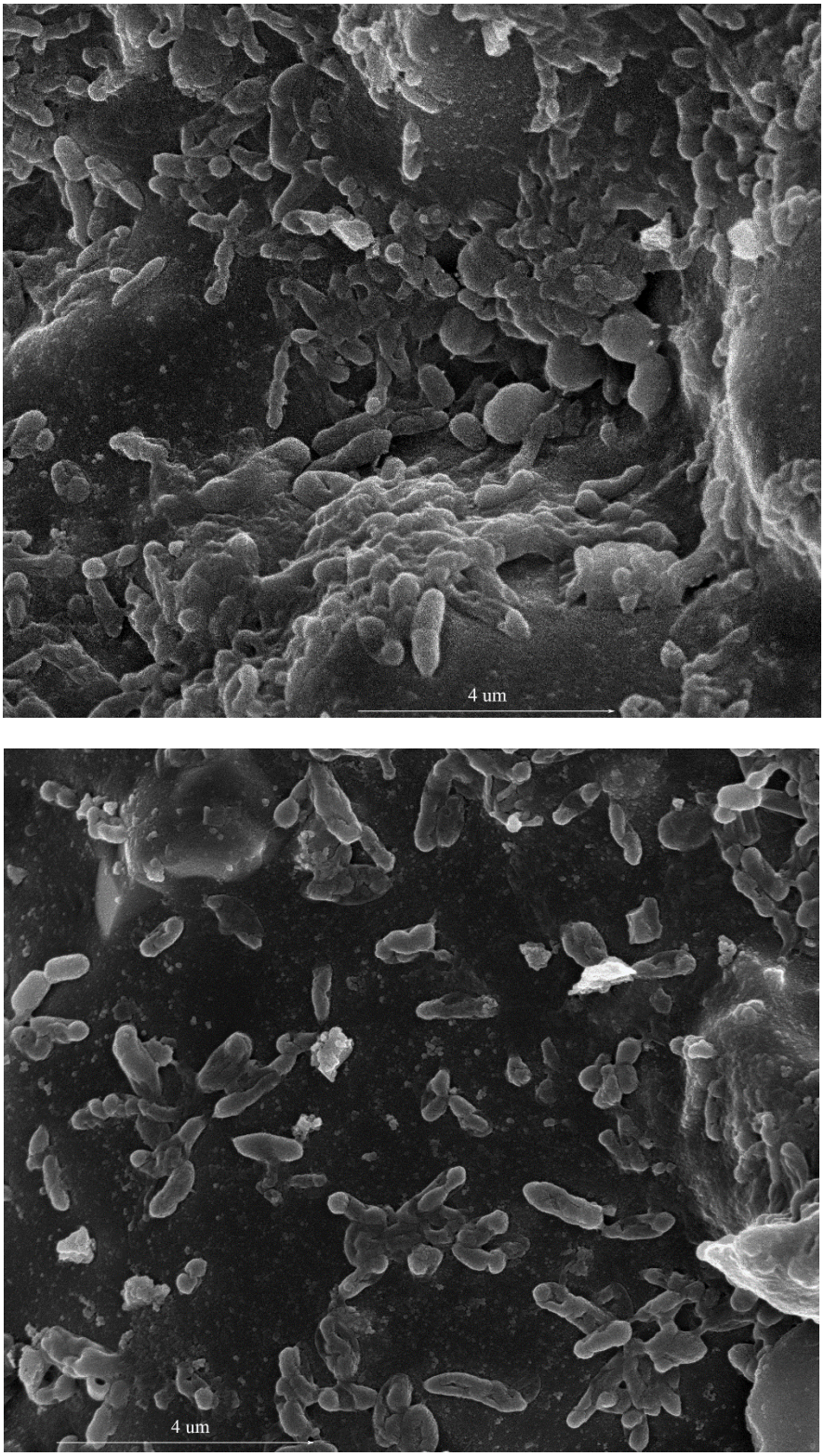

Figure 4.106: FESEM image of biofilm formation during the biodegradation of 3-CP by the defined mixed consortium in PBBR-2.

4.10. Biodegradation of 4-chlorophenol in airlift inner loop bioreactor (ALR)

The present study focussed on the continuous biodegradation of 4-chlorophenol in an airlift inner loop bioreactor (ALR) using the mixed microbial consortium. During the continuous operation, the effect of different parameters such as hydraulic retention time, loading rate, biogenic substrate concentration and initial substrate concentration on removal efficiency of ALR has been studied. The different metabolites present in the effluent were detected to analyze the biodegradation pathway followed by the mixed consortium.

Airlift reactors (ALR) are simple in design with no moving parts or agitator, thus economical also. In ALR, pressurized gas is used for internal mixing and aeration. Also, ALR has low shear stress on microbial cells which will be an advantage for shear sensitive cells. Packed bed reactor where usually mass and oxygen transfer limitation and clogging of packing bed occurs, in ALR these problems can be avoided [21, 24]. There are several reports on biodegradation of chlorophenols and other toxic compounds using ALR. Xiangchun et al., (2003, 2004) reported the removal of phenol and 2,4-dichlorophenol in the airlift honeycomb like ceramic reactor [21, 100]. They have used ceramic honeycomb-like structure placed inside the riser for immobilization of pure culture *Achromobacter* sp. A packed bed bioreactor equipped with the net draft tube riser (PB-ALR) for liquid circulation and oxygenation, was constructed for the removal of 2,4,6-trichlorophenol [22]. In the present study, simple airlift bioreactor without any immobilization of microorganism was used for removal of 4-chlorophenol. The absence of agitator for mixing and any carrier for immobilization of microorganism makes the bioreactor simple with ease in operation and economical.

The bioreactor was operated for 60 days in a continuous mode, divided into three different parts. The first part comprises of the study of the effect of peptone concentration on removal efficiency of 4-CP. Second, the effect of substrate concentration was studied by gradually increasing it and keeping the other parameters constant. The last part was focussed on the effect of HRT on 4-CP removal efficiency. The HRT was gradually decreased by keeping the 4-CP and peptone concentration constant. The bioreactor showed excellent removal of 4-chlorophenol during continuous operation.

Before the commencement of the continuous operation, the reactor was operated without inoculation to check the abiotic loss of the 4-CP. The bioreactor was filled with MSM containing 40 mg/L of 4-CP for oneday, and the air flow was set to 4 LPM. Only 2-3% of the abiotic loss was observed at this conditions that is negligible as compared to biodegradation.

The bioreactor was filled with 12 L MSM containing 20 mg/L of 4CP and inoculated with 10 %(v/v) mixed consortium. The bioreactor was operated in the fed-batch mode before the continuous study to acclimatize the biomass. The 4-CP was added to bioreactor periodically to maintain the 20 mg/L concentration. This acclimatization process was performed for one week after which the continuous operation was started.

4.10.1. Effect of Peptone

The effect of peptone on the removal of 4-CP in ALR was studied throughout the operation. The bioreactor started with 1 g/L peptone with 40 h HRT. The effects of peptone on the removal of 4-CP by ALR are presented in figure 4.107 and 4.108. About 99% of the 20 mg/L of initial 4-CP present in the influent had been removed. The removal efficiency decreased to around 80% at 40 h HRT on increasing the initial substrate concentration to 40 mg/L. Further, decrease in HRT to 30 h, the removal efficiency decreased to around 50%, which again increased during steady state operation and stabled at 60% with 1 g/L peptone. Initially biomass increased with time and concentration at 40 h HRT. But when the HRT decreased to 30 h, washout occurred and consequently biomass concentration in the reactor decreased (Figure 4.107).

At this stage, on 15th day of the operation, the peptone concentration was decreased to 0.5 g/L in the influent by keeping initial 4-CP concentration at 40 mg/L and HRT at 30 h (Figure 4.107). Surprisingly, the removal efficiency of the bioreactor was increased from 60% to 76% in the presence of low peptone concentration. The removal efficiency of reactor got stable at steady state condition. After a steady state condition, the peptone concentration was further decreased to 0.2 g/L in the influent on the 27th day of the operation. The HRT was set to 48 h and 4-CP concentration in the influent was increased gradually at this condition. The bioreactor showed 100% removal of 40 mg/L of 4-CP. The bioreactor performance increased greatly in the presence of 0.2 g/L peptone. Even the bioreactor was able to remove up to 400 mg/L of 4-CP in the influent with greater than 98% removal efficiency. Also, it was observed that the biomass concentration increased in the reactor when peptone concentration was reduced. The results indicated that the mixed consortium could utilize the 4-CP as a carbon and energy source with complete mineralization. Also, the presence of a higher concentration of peptone interferes the degradation of 4-CP by the mixed consortium. However, the presence of 0.2 g/L of peptone is essential for the removal of 4-CP as concluded from the batch study (data not shown). The batch study showed that the presence of 0.2 g/L of peptone improves the degradation efficiency compared to the absence of peptone. The possible reason is that peptone may serve as a nitrogen source to the mixed consortium as it was used during the acclimatization period. Also, the peptone didn't contribute to biomass growth as evidenced by the result that, the biomass concentration increased with the decrease in peptone concentration and increase in 4-CP concentration. Thus, it could be concluded that the mixed consortium had utilized the 4-CP as carbon source only. Peptone concentration should be kept at 0.2 g/L for achieving maximum removal rate. Table 4.27 summarizes the effect of peptone on the removal rate of 4-CP by the mixed consortium.

Only a specific group of microorganism in the mixed consortium are responsible for the degradation of 4-CP only. Sahinkaya and Dilek (2006) studied the effect of peptone on the degradation of 4-CP in sequencing batch reactor. They also reported that the competent biomass (specialist) is responsible for 4-CP degradation and so the specific degradation rate of 4-CP

increases with decreasing peptone concentration as the fraction of competent biomass increased in the mixed consortium [16].

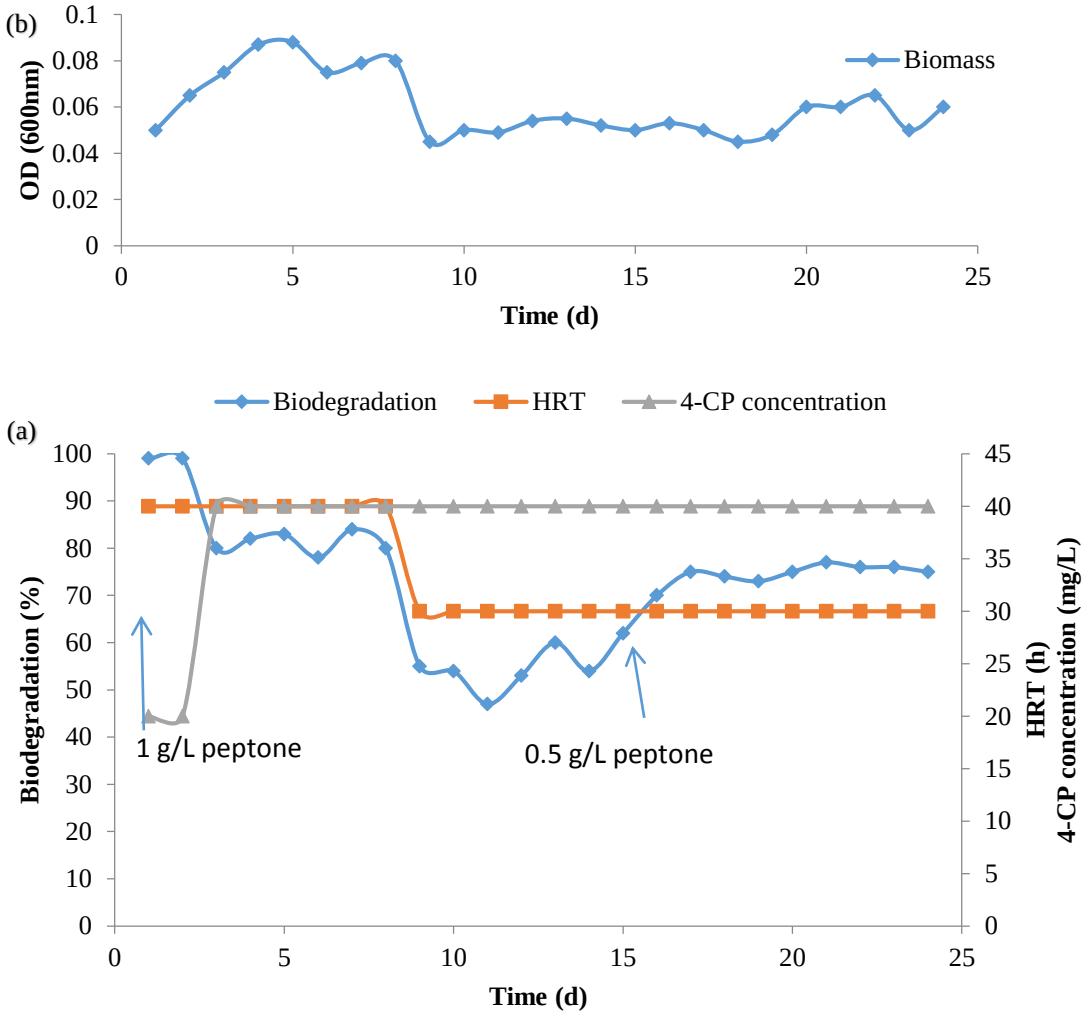

Figure 4.107: (a) 4-CP removal by ALR and the effect of peptone concentration. (b) The change in biomass concentration in the effluent during the continuous operation.

Table 4.27: Effect of peptone concentration on 4-CP removal by ALR

Time (d)	Influent Concentration	HRT ±1 (h)	Loading rate (mg/L/d)	Peptone (mg/L)	Biodegradation (%)	Volumetric removal rate (mg/L/h)
1-2	20	40	12	1	99	0.495
3-8	40	40	24	1	82	0.82
9-14	40	30	32	1	56.5	0.753
15-24	40	30	32	0.5	75.8	1.01

29-31	40	48	20	0.2	100	0.84
42-43	200	48	100	0.2	100	4.17
44	250	48	125	0.2	99	5.2

Effect of peptone on the degradation of 4-CP showed than 0.2 g/L peptone is essential for maximum degradation and removal efficiency. There are several theories that can apply here. At high peptone concentration, the removal efficiency was observed to decreased may due to in the presence of peptone, the fraction of other microorganism increases compares to competent biomass responsible for 4-CP degradation. Also peptone is a microbial easily degradable substrate, so the microbes may utilize the peptone first rather than 4-CP.

4.10.2. Effect of substrate concentration

Effect of initial 4CP concentration on the removal efficiency of the bioreactor was studied by keeping the HRT (48±1 h) and peptone concentration (0.2 g/L) constant and the 4-CP concentration was gradually increased. The bioreactor achieved greater than 99% removal efficiency when the initial 4-CP concentration was increased from 20 to 400 mg/L. The effect of initial substrate concentration on biodegradation is shown in Figure 4.108 (Day 27 to 47). After 150 mg/L, the 4-CP concentration was increased at a fast rate (150 to 400 mg/L) to check the bioreactor stability for shock loads. The bioreactor showed great stability and removal efficiency even during the shock loads. At lower concentration of 4-CP, complete mineralization was observed with no traces of the intermediate compounds. However at higher concentration (above 250 mg/L), the trace amount of 5-chloro 2-hydroxymuconic semialdehyde (5-CHMS) has been observed. The 5-CHMS (λmax= 380 nm) is the meta-cleavage product of the 4-chlorocatechol that gives characteristic yellow color in the medium. When 4-CP concentration was increased to 400 mg/L, the medium in the bioreactor was turned to light yellow color (Figure 3.8b). The biomass concentration in the effluent was also observed to increase with increasing 4-CP concentration. These results indicated that the microorganisms were able to utilize the 4-CP as a carbon and energy source.

4.10.3. Effect of HRT

The effect of HRT on the removal of 4-CP by ALR was studied from day 47 to 60. The peptone and initial 4-CP concentration was kept constant at 0.2 and 400 g/L, respectively. The change in biodegradation of 4-CP at different HRT are shown in figure 4.108. When the HRT gradually decreases from 48 h to 24 h, no abrupt change in the removal efficiency was observed. The bioreactor achieved greater than 99% removal of 4-CP at up to 24 h HRT. The presence of 5-CHMS was not observed in the effluent at higher HRT. However further decrease in HRT to 18 h lead to a drastic decrease in biodegradation to 50%. At 18 h HRT, washout occurred as evidenced by a sudden decrease in biomass concentration in effluent and also the bioreactor medium had been found to turn into light brown color from light yellow color. The concentration of 4-CP in the effluent increased suddenly. Effect of different HRT on the volumetric removal

rate of 4-CP (400 mg/L) is shown in figure 4.109. The maximum volumetric removal rate observed was at 24 h HRT as shown in figure 4.109. To regain the biodegradation capacity of the reactor, the HRT was again increased stepwise to 24 h and 30 h. The bioreactor was took some time to recover full degradation capacity. At 24 h HRT, the bioreactor showed only slight increase in biodegradation. The HRT was increased to 30 h and after two days, the biodegradation was observed to increase gradually to 66%. After washout, the bioreactor was not able to recover back its full biodegradation ability immediately as it was observed before. However with time and increase in HRT, the bioreactor had shown to regain biodegradation capacity once again. Table 4.28 summarizes the change in volumetric removal rate with HRT and initial 4-CP concentration.

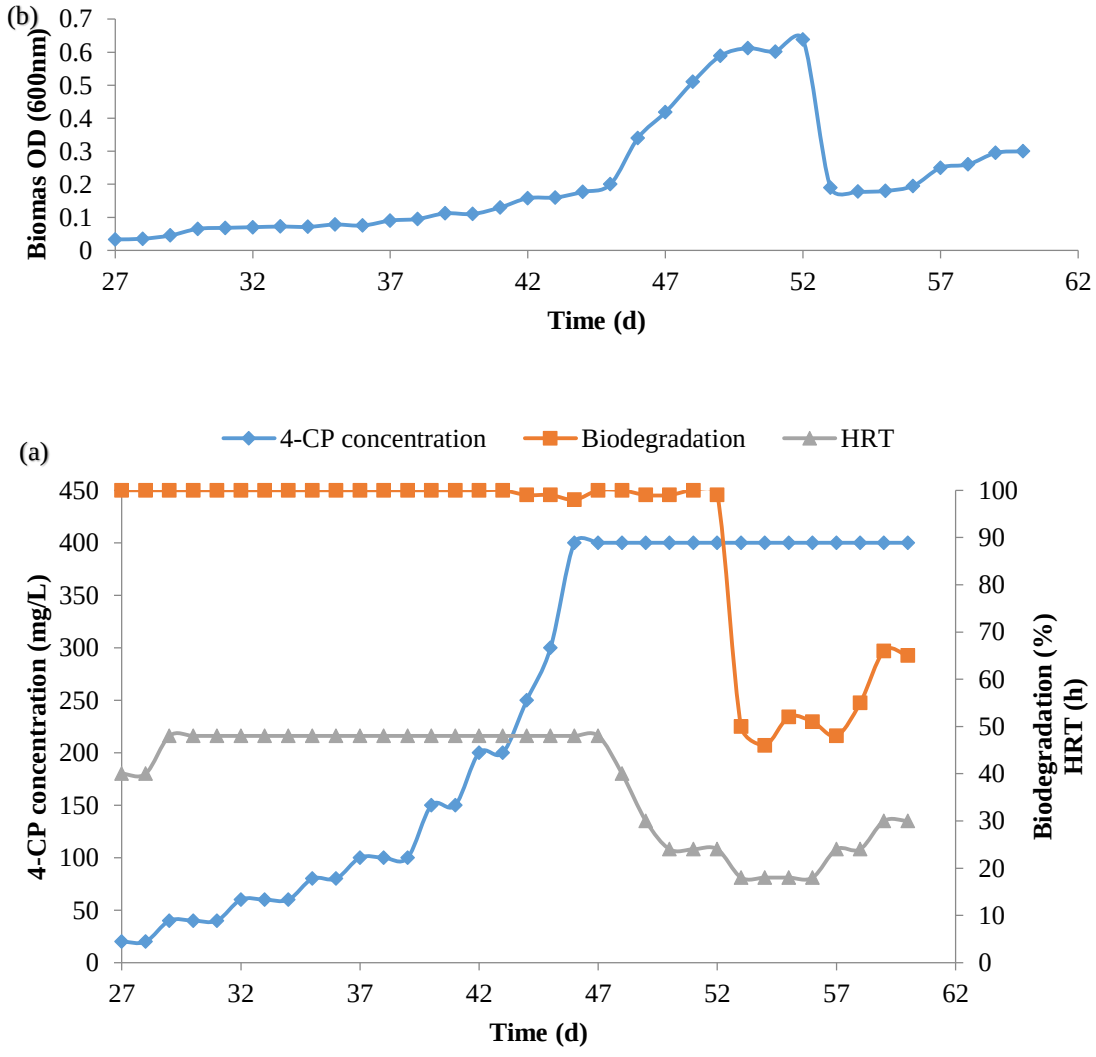

Figure 4.108: (a) 4-CP removal efficiency by ALR in the presence of 0.2 g/L peptone and effect of initial substrate concentration and HRT. (b) The change in biomass concentration in the effluent during the continuous operation.

The relationship between volumetric loading rate and volumetric removal rate is depicted in figure 4.110. The removal rate was exponentially increased with increasing loading rate up to 16.67 mg/L/h, where the removal rate observed was 16.59 mg/L/h. When loading rate increases to 22.25 mg/L/h, the removal rate was decreased to 11.13 mg/L/h. So, to obtain the maximum or greater than 98% removal rate the HRT should be kept at 24 h and loading rate at 16.67 mg/L/h. Kargi and konya (2007) studied the effect of HRT on the removal of 4-CP in activated sludge unit. They have reported volumetric removal rate in the range of 360 to 720 mg/L/d with high COD for different HRT of 5 to 15 h. After 15 h of HRT, no significance increase in the removal rate was observed [234]. In another study, it was reported the degradation of 4-CP by UASB was decreased with a decrease in HRT. The UASB has shown 90.1, 88.3, 84.6, and 83% degradation of 4-CP at 16, 12, 8, and 6 h respectively [150]. Table 4.29 shows the performance of different bioreactors for removal of chlorophenols. The most of the studies have shown the removal of 4-CP below 200 mg/L/d of loading rate. Kargi and Konya, (2007) showed the removal of 4-CP up to 800 mg/L/d of loading rate with 90% efficiency [234]. The present study has shown the removal of 99.8% for higher loading rate of 400 mg/L/d. The performance of the ALR for removal of 4-CP is prominent and could be successfully applied for contaminated wastewater treatment.

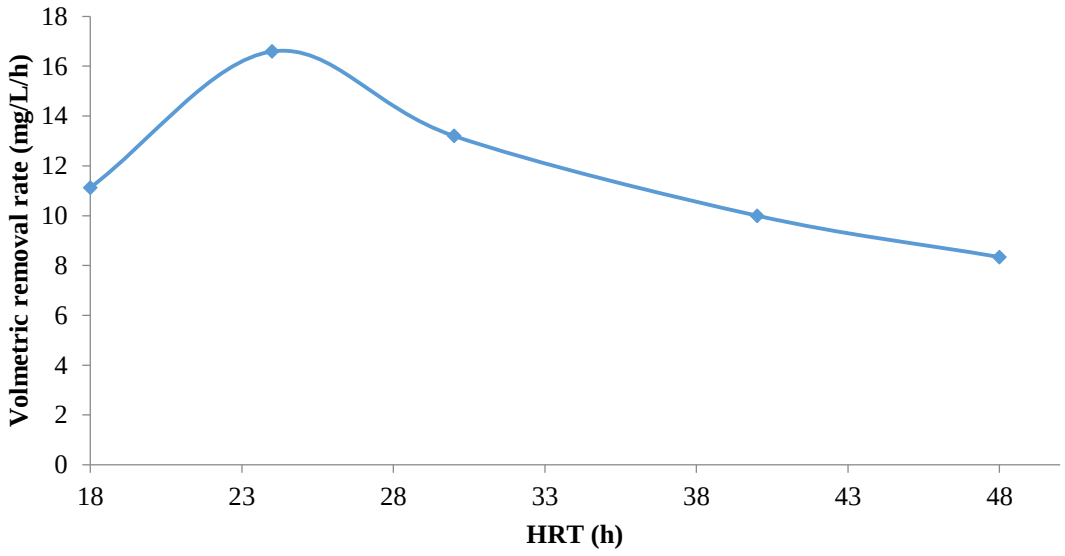

Figure 4.109: Effect of HRT on the volumetric removal rate of 4-CP (400 mg/L) in the ALR with 0.2 g/L of peptone.

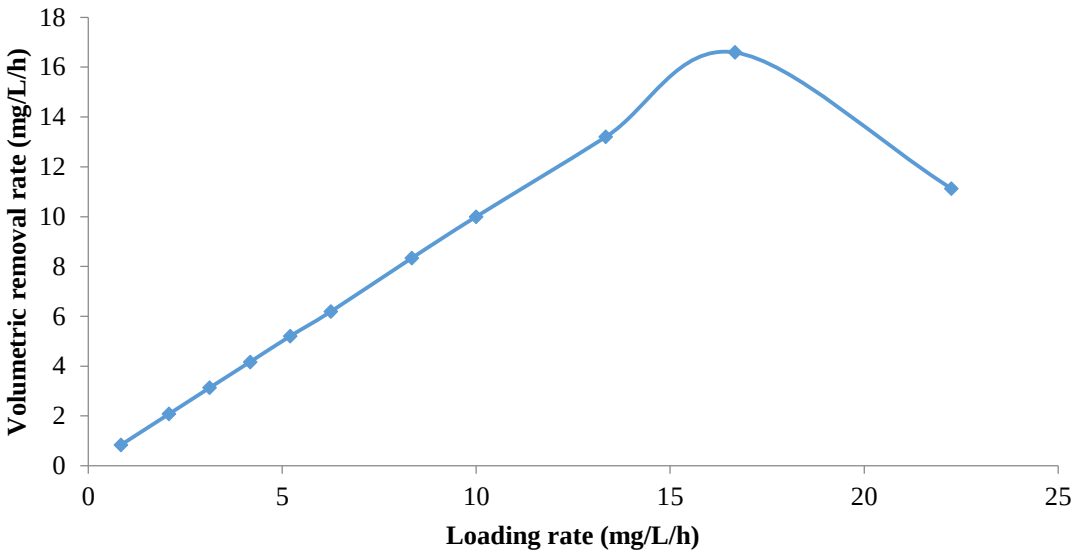

Figure 4.110: Effect of loading rate on the volumetric removal of 4-CP in the ALR with 0.2 g/L of peptone.

Table 4.28: Removal of 4-CP by ALR in the presence of 0.2 g/L peptone.

Time (d)	Influent Concentration	HRT ±1 (h)	Loading rate (mg/l/d)	Biodegradation (%)	Volumetric removal rate (mg/L/h)
45	300	48	150	99	6.19
46-47	400	48	200	100	8.34
48	400	40	240	100	10
49	400	30	320	99	13.2
50-52	400	24	400	99.5	16.59
53-56	400	18	534	50	11.13

4.10.4. Metabolites

During the biodegradation of 4-CP by airlift inner loop reactor, the spectroscopic and HPLC analysis indicates the presence of 5-CHMS in the effluent. At lower concentration of 4-CP, no metabolites had been detected in the effluent. However at higher 4-CP concentration, the presence of 5-CHMS (λmax= 380 nm) was detected. This was also evidenced by the appearance of light yellow color in the reactor medium. 5-CHMS is the meta-cleavage product of the 4-chlorocatechol, the first intermediate of the 4-CP degradation pathway. When 4-CP concentration is above 300 mg/L, the calorimetric assay of the effluent sample shows the presence of 4-chlorocatechol. The present of metabolites shows that the mixed consortium

follows the *meta*- cleavage pathway for 4-CP biodegradation. There were no other metabolites detected in the effluent. These results were also confirmed in the batch study. The degradation of 4-CP via *meta*- cleavage pathway by the mixed consortium and pure microorganism have been reported [40, 49].

The bioreactor showed great removal efficiency throughout the operation with greater than 98% removal of 4-CP. The optimum hydraulic retention time for the bioreactor was found to be 24 h and below that the washout of the microbes observed. In the presence of higher peptone, concentration removal efficiency was observed to decrease, and the optimum 0.2 g/L of peptone was essential for the maximum removal efficiency of 4-CP. The maximum initial substrate concentration of 400 mg/L of 4-CP was degradedup to 99% at 24 h HRT and 0.2 g/L peptone. The maximum volumetric removal rate obtained was 16.59 mg/L/h at 24 h HRT and 400 mg/L/h of 4-CP. The presence of 5-CHMS at a higher 4-CP concentration in the bioreactor establishes that the mixed consortium follows the meta- cleavage pathway.

Table 4.29: Biodegradation of chlorophenols using different bioreactors

Bioreactor	Compound	Concentration	HRT (h)	Loading rate (mg/L/d)	Removal rate (%)	Reference
FBR	4-CP	99.13	24.4	97.5	98.7	[139]
PBR	4-CP	20	2.78	172	100	[17]
CSTR	4-CP	20	4.17	115	100	[17]
UASB	4-CP	40	12	80	88.3	[150]
ASU	4-CP	500	15	800	90	[234]
ALR	2,4-DCP	7-103	6.25	26-394	100-88	[21]
ALR	2,4-DCP	28.5	8	85.5	97.8	[100]
PB-ALR	2,4,6-TCP	5.76	2.95	46.88	99.9	[22]
ALR	4-CP	400	24	400	99.8	This study

Activated sludge unit consisted of an aeration tank and a sludge settling tank (ASU); Fluidized bed reactor (FBR); Packed bed reactor (PBR); Continuous stirred tank reactor (CSTR); Upflow anaerobic sludge blanket (UASB); Airlift reactor (ALR); Packed bed airlift reactor (PB-ALR)

Summary:

This section discussed the summary of four parts 4.7, 4.8, 4.9 and 4.10 i.e. continuous biodegradation of chlorophenols by pure and mixed microbial consortium in the bioreactors.

- Both pure and mixed cultures, isolated in present study, showed potential for continuous biodegradation of chlorophenols in the bioreactor.
- Ceramic beads were found suitable material for support to attach microorganism to form a biofilm.
- **Effect of peptone:** There was no drastic change in chlorophenols biodegradation was observed when peptone concentration was reduced, except 3-CP in which higher peptone concentration is required for the higher degradation rate. The biodegradation of 2,4-DCP (PBBR-1) and 4-CP (ALR and PBBR-2) showed that the microorganism were able to utilize them as a carbon and energy source (both pure and mixed culture) based on the fact that reducing peptone concentration did not affect the biomass growth. Also, the biomass growth increased with increasing substrate concentration. While in case of 3-CP, the reverse effect was observed in which higher peptone concentration supports the higher biodegradation rate of 3-CP by mixed culture.
- **Effect of HRT:** Biodegradation of chlorophenols in three different bioreactor system showed that HRT has a great influence on the removal rate. In case of PBBR-1 (2,4-DCP) and PBBR-2 (4-CP), it was observed that the volumetric removal rate increases with decreasing HRT. While in case of PBBR-2 (3-CP) and ALR (4-CP), the volumetric removal rate increases with decreasing HRT up to certain value and after that the removal rate started decreasing. The optimum HRT for biodegradation of different chlorophenols varies according to the toxicity of the compound, microorganism growth rate, biodegradation rate and presence of biogenic substrate. However, at higher HRT, it was observed that the biodegradation rate increases leading to complete removal of chlorophenols. At lower HRT, washout of the microbes and inhibition of the microbial growth was observed leading to decreased biodegradation rate.
- **Effect of loading rate:** In case of loading rate or initial substrate concentration, the effects on biodegradation and removal of chlorophenols varies with the toxicity of the compound and presence of biogenic substrate considering HRT constant. The removal rate of chlorophenols was increased with increasing loading rate up to certain value and after that no more improvement in degradation rate or removal rate observed. The result indicated that the biodegradation rate was decreased at higher loading rate due to increased toxicity.
- The effect of three different parameters i.e. HRT, loading rate and biogenic substrate (peptone) concentration, were evaluated in detail on biodegradation of chlorophenols by pure and mixed culture in a bioreactor. It was concluded that optimization of these parameters results in two different effects i.e. maximum volumetric removal rate and maximum biodegradation rate. In most of the treatment facility the goal was to achieve maximum biodegradation rate (complete biodegradation) before wastewater is release into the mainstream. Table presented below shows the optimum condition obtained in the

present study for achieving maximum removal rate and biodegradation of different chlorophenols.

Comparative study showing optimum conditions for achieving maximum removal rate and degradation rate in the bioreactor

Reactor	Compound	HRT	Peptone (mg/L)	Loading rate (mg/L/d)	Vol. removal rate (mg/L/d)	Degradation rate (%)
Maximum removal rate efficiency (mg/L/d)						
PBBR-1	2,4-DCP	12.5	0.2	172.8	127.18	73.6
PBBR-2	3-CP	30	0.2	48	36.48	76
PBBR-2	4-CP	30	0.2	72	52.56	73
ALR	4-CP	24	0.2	400	400	99.8
PBBR-1	2,4-DCP	12.5	1	115.2	109.44	95
PBBR-2	3-CP	30	1	56	54.88	98
PBBR-2	4-CP	15	1	64	42.24	66
ALR	4-CP	40	1	24	19.68	82
Maximum biodegradation rate efficiency (%)						
PBBR-1	2,4-DCP	12.5	0.2	115.2	113	98
PBBR-2	3-CP	30	1	56	55.5	99
PBBR-2	4-CP	30	0.2	32	30.1	94
ALR	4-CP	24	0.2	400	400	99.8

CONCLUSIONS AND RECOMNENDATIONS

CONCLUSIONS

- Four different pure bacterial strains were successfully isolated and selected for biodegradation of chlorophenols based on their high tolerance level and degradation capability. The microbes of interest were characterized morphologically, biochemically as well as molecularly and were identified to be *Bacillus cereus*, *Bacillus endophyticus*, *Kocuria rhizophila* and *Pseudomonas aeruginosa*. The 16S rDNA sequence of the strains was indexed in GenBank database of NCBI for future reference.
- The four experimental parameters i.e. pH, temperature, inoculum size and ammonium sulphate concentration, were efficiently optimized using Response Surface Methodology. At optimized conditions, the biodegradation rate and tolerance level of the pure culture increased significantly.
- Biodegradation of five chlorophenols including 2-CP, 3-CP, 4-CP, 2,4-DCP, and 2,4,6-TCP were successfully performed by the pure and mixed cultures. The pure strains have the ability to degrade 2-CP and 2,4-DCP even for high concentration, however, their degradation rate greatly diminished for 3-CP and 4-CP. None of the individual strains was able to degrade 2,4,6-TCP and PCP. All the four strains were unable to utilize 3-CP, 4-CP, and 2,4,6-TCP individually. However, in the mixed consortium they showed substantial degradation of all the three monochlorophenols.
- The mixed microbial consortium, both defined and undefined, showed complete degradation of 3-CP, 4-CP but not 2-CP. The defined mixed consortium have shown degradation of 2,4,6-TCP after an acclimation process, but the undefined mixed consortium failed to degrade it even after acclimation.
- PCP has shown highest resistance and neither pure nor mixed cultures were able to utilize PCP. However, defined consortium has shown PCP degradation at low concentration in the presence of peptone.
- Multi-substrate biodegradation of chlorophenols by pure and mixed cultures has shown the great potential of isolated microorganism for *in-situ* bioremediation. The mixed microbial consortium, especially defined consortium had shown significant results for biodegradation of mixture of chlorophenols. Cometabolism results showed that the presence of lower chlorophenols increased the total chlorophenol removal rate in both pure and mixed culture. In case of pure culture and defined mixed culture, the 2-CP contributes the increased degradation of higher chlorophenols and total chlorophenol degradation rate. While in case of undefined mixed culture, 3-CP and 4-CP contributed the increased degradation rate of higher chlorophenols and total chlorophenols rate.
- PCP showed highest resistant and neither pure nor mixed cultures were able to utilize PCP. However, defined consortium has shown PCP degradation at low concentration in the presence of peptone.

- ALR showed excellent removal of 4-CP by the mixed consortium (undefined) in continuous mode. The maximum removal rate achieved was 16.59 mg/L/h for 4-CP at 0.2 g/L peptone and 24 h HRT.
- PBBR showed removal of 2,4-DCP by pure strains and removal of 3-CP and 4-CP by the defined mixed consortia. In PBBR-1, maximum volumetric removal rate achieved for was 127.2 mg/l/d 2,4-DCP at a loading rate of 178.8 mg/L/d with 0.2 g/L of peptone and 12.5 h HRT. While in PBBR-2, maximum volumetric removal rate achieved for 4-CP was 52.56 mg/L/d at 72 mg/L/d loading rate and for 3-CP was 36.48 mg/L/d at 48 mg/L/d loading rate.
- The biofilm formation improved the biodegradation efficiency of the microbes and prevents washout at high flow rate.
- Overall, ALR had shown the significant result for 4-CP removal than PBBR and is more efficient for chlorophenol removal.

FUTURE RECOMMENDATIONS

Although a significant amount of work has been done on the biodegradation of chlorophenols, there are still many areas that require more research attention.

- A better understanding of the metabolic pathways and the enzymes involved is required for achieving improved bioremediation efficiency.
- The direct application of enzymes for bioremediation is another field of research that requires attention.
- Similarly, application of microorganism for *in-situ* bioremediation necessitates more research studies of microorganism under extreme conditions such as temperature, pH, soil condition, etc.
- The use of genetic engineering for producing recombinant microorganism that can degrade mixed chlorophenols with other toxic compounds simultaneously in the environment will play a significant role in future.
- Studies pertaining combination of physiochemical with biological treatment as well as the anaerobic and aerobic reaction is significant to achieve complete bioremediation.
- Recently energy production from the biodegradation of toxic compounds such as microbial fuel cell is gaining importance among the researcher. Hence, a study in this direction may be double beneficial to the society.

REFERENCES

REFERENCES

[1] ATSDR, Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), Priority List of Hazardous Substances, (2011).
[2] C.J. Snyder, M. Asghar, J.M. Scharer, R.L. Legge, Biodegradation kinetics of 2,4,6-trichlorophenol by an acclimated mixed microbial culture under aerobic conditions, Biodegradation, 17 (2006) 535-544.
[3] A.O. Olaniran, E.O. Igbinosa, Chlorophenols and other related derivatives of environmental concern: properties, distribution and microbial degradation processes, Chemosphere, 83 (2011) 1297-1306.
[4] P.A. Demers, H.W. Davies, M.C. Friesen, C. Hertzman, A. Ostry, R. Hershler, K. Teschke, Cancer and occupational exposure to pentachlorophenol and tetrachlorophenol (Canada), Cancer Causes Control, 17 (2006) 749-758.
[5] E.O. Igbinosa, E.E. Odjadjare, V.N. Chigor, I.H. Igbinosa, A.O. Emoghene, F.O. Ekhaise, N.O. Igiehon, O.G. Idemudia, Toxicological profile of chlorophenols and their derivatives in the environment: the public health perspective, ScientificWorldJournal, 2013 (2013) 460215.
[6] IARC, IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans: Some Halogenated Hydrocarbons and Pesticide Exposures, vol. 41, International agency for research on cancer, Lyon, France, (1986).
[7] G.S. Cooper, S. Jones, Pentachlorophenol and cancer risk: focusing the lens on specific chlorophenols and contaminants, Environ Health Perspect, 116 (2008) 1001-1008.
[8] U.G. Ahlborg, T.M. Thunberg, Chlorinated phenols: occurrence, toxicity, metabolism, and environmental impact, Crit Rev Toxicol, 7 (1980) 1-35.
[9] V. Daniel, W. Huber, K. Bauer, C. Suesal, J. Mytilineos, A. Melk, C. Conradt, G. Opelz, Association of elevated blood levels of pentachlorophenol (PCP) with cellular and humoral immunodeficiencies, Arch Environ Health, 56 (2001) 77-83.
[10] M. Czaplicka, Sources and transformations of chlorophenols in the natural environment, Sci Total Environ, 322 (2004) 21-39.
[11] Y. Herrera, A. Okoh, L. Alvarez, N. Robledo, M. Trejo-Hernández, Biodegradation of 2,4-dichlorophenol by a Bacillus consortium, World Journal of Microbiology and Biotechnology, 24 (2008) 55-60.
[12] J. Field, R. Sierra-Alvarez, Microbial degradation of chlorinated phenols, Reviews in Environmental Science and Bio/Technology, 7 (2008) 211-241.
[13] Z. Hu, R.A. Ferraina, J.F. Ericson, B.F. Smets, Effect of long-term exposure, biogenic substrate presence, and electron acceptor conditions on the biodegradation of multiple substituted benzoates and phenolates, Water Res, 39 (2005) 3501-3510.
[14] D. De Los Cobos-Vasconcelos, F. Santoyo-Tepole, C. Juárez-Ramírez, N. Ruiz-Ordaz, C.J.J. Galíndez-Mayer, Cometabolic degradation of chlorophenols by a strain of Burkholderia in fed-batch culture, Enzyme and Microbial Technology, 40 (2006) 57-60.
[15] M. Goel, M. Ramesh, T. Sreekrishnan, Mixed Culture Acclimatization and Biodegradation of Chlorophenols in Shake Flasks: Effect of the Inoculum Source, Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management, 13 (2009) 29-34.
[16] E. Sahinkaya, F.B. Dilek, Effect of biogenic substrate concentration on 4-chlorophenol degradation kinetics in sequencing batch reactors with instantaneous feed, J Hazard Mater, 137 (2006) 282-287.
[17] J.-H. Kim, K.-K. Oh, S.-T. Lee, S.-W. Kim, S.-I. Hong, Biodegradation of phenol and chlorophenols with defined mixed culture in shake-flasks and a packed bed reactor, Process Biochemistry, 37 (2002) 1367-1373.

[18] I. Durruty, E. Okada, J. González, S. Murialdo, Multisubstrate monod kinetic model for simultaneous degradation of chlorophenol mixtures, Biotechnology and Bioprocess Engineering, 16 (2011) 908-915.
[19] M. Alexander, Biodegradation and Bioremediation, Academic press, San Diego, 1999.
[20] H.S. Bae, J.M. Lee, S.-T. Lee, Biodegradation of the mixture of 2, 4, 6-trichlorophenol, 4-chiorophenol, and phenol by a defined mixed culture, J Gen Appl Microbiol, 43 (1997) 97-103.
[21] X. Quan, S. Hanchang, Z. Yongming, W. Jianlong, Q. Yi, Biodegradation of 2,4-dichlorophenol in an air-lift honeycomb-like ceramic reactor, Process Biochemistry, 38 (2003) 1545-1551.
[22] A. Gomez-De Jesus, F.J. Romano-Baez, L. Leyva-Amezcua, C. Juarez-Ramirez, N. Ruiz-Ordaz, J. Galindez-Mayer, Biodegradation of 2,4,6-trichlorophenol in a packed-bed biofilm reactor equipped with an internal net draft tube riser for aeration and liquid circulation, J Hazard Mater, 161 (2009) 1140-1149.
[23] E.I. Atuanya, T. Chakrabarti, Kinetics of biotransformation of 2,4-dichlorophenol using UASB-reactor, Environ Monit Assess, 96 (2004) 129-141.
[24] H. Zilouei, B. Guieysse, B. Mattiasson, Biological degradation of chlorophenols in packed-bed bioreactors using mixed bacterial consortia, Process Biochemistry, 41 (2006) 1083-1089.
[25] EPA, Ambient Water Quality Criteria for Chlorinated Phenols. US Environmental Protection Agency, Washington, D.C., (1980).
[26] G.F. Fries, V.J. Feil, R.G. Zaylskie, K.M. Bialek, C.P. Rice, Treated wood in livestock facilities: relationships among residues of pentachlorophenol, dioxins, and furans in wood and beef, Environ Pollut, 116 (2002) 301-307.
[27] IARC, . IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: OveraIl Evaluations of Carcinogenicity, Supplement 7, IARC, Lyon, France, (1987).
[28] IARC, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Occupational Exposures in Insecticide Application, and Some Pesticides, vol. 53, IARC, Lyon, France, (1991).
[29] G. Collins, C. Foy, S. McHugh, V. O'Flaherty, Anaerobic treatment of 2,4,6-trichlorophenol in an expanded granular sludge bed-anaerobic filter (EGSB-AF) bioreactor at 15 degrees C, FEMS Microbiol Ecol, 53 (2005) 167-178.
[30] EPA, National Primary Drinking Water Regulation: Drinking Water Contaminants, US Environmental Protection Agency, 816-F-09-0004, (2009).
[31] WHO, Guidelines for drinking-water quality - vol. 1: Recommendations 3rd ed. World Health Organization, Geneva, (2008).
[32] ATSDR, Toxicological profile for Chlorophenols. U.S. Department of Health and Human Services, Atlanta, GA, (1999).
[33] K. Becker, C. Schulz, S. Kaus, M. Seiwert, B. Seifert, German Environmental Survey 1998 (GerES III): environmental pollutants in the urine of the German population, Int J Hyg Environ Health, 206 (2003) 15-24.
[34] R.H. Hill, Jr., S.L. Head, S. Baker, M. Gregg, D.B. Shealy, S.L. Bailey, C.C. Williams, E.J. Sampson, L.L. Needham, Pesticide residues in urine of adults living in the United States: reference range concentrations, Environ Res, 71 (1995) 99-108.
[35] J. Michałowicz, The Occurrence of Chlorophenols, Chlorocatechols and Chlorinated Methoxyphenols in Drinking Water of the Largest Cities in Poland, Pol J Environ Stud, 14 (2005) 327-333.
[36] K. Pekari, M. Luotamo, J. Jarvisalo, L. Lindroos, A. Aitio, Urinary excretion of chlorinated phenols in saw-mill workers, Int Arch Occup Environ Health, 63 (1991) 57-62.

[37] H. Mussalo-Rauhamaa, H. Pyysalo, K. Antervo, The presence of chlorophenols and their conjugates in Finnish human adipose and liver tissues, Sci Total Environ, 83 (1989) 161-172.
[38] R.E. Cline, R.H. Hill, Jr., D.L. Phillips, L.L. Needham, Pentachlorophenol measurements in body fluids of people in log homes and workplaces, Arch Environ Contam Toxicol, 18 (1989) 475-481.
[39] M.M. Haggblom, Microbial breakdown of halogenated aromatic pesticides and related compounds, FEMS Microbiol Rev, 9 (1992) 29-71.
[40] A. Farrell, B. Quilty, Degradation of mono-chlorophenols by a mixed microbial community via a meta- cleavage pathway, Biodegradation, 10 (1999) 353-362.
[41] M. Häggblom, Mechanisms of bacterial degradation and transformation of chlorinated monoaromatic compounds, J Basic Microbiol, 30 (1990) 115-141.
[42] P. Arora, H. Bae, Bacterial degradation of chlorophenols and their derivatives, Microb Cell Fact, 13 (2014) 31.
[43] K. Veenagayathri, N. Vasudevan, Ortho and meta cleavage dioxygenases detected during the degradation of phenolic compounds by a moderately halophilic bacterial consortium, Inter Res J Microbio, 2 (2011) 406-414.
[44] E. Schmidt, M. Hellwig, H.J. Knackmuss, Degradation of chlorophenols by a defined mixed microbial community, Appl Environ Microbiol, 46 (1983) 1038-1044.
[45] I. Bartels, H.-J. Knackmuss, W. Reineke, Suicide Inactivation of Catechol 2,3-Dioxygenase from Pseudomonas putida mt-2 by 3-Halocatechols, Appl Environ Microbiol, 47 (1984) 500-505.
[46] G.M. Klecka, D.T. Gibson, Inhibition of catechol 2,3-dioxygenase from Pseudomonas putida by 3-chlorocatechol, Appl Environ Microbiol, 41 (1981) 1159-1165.
[47] M. Wieser, J. Eberspächer, B. Vogler, F. Lingens, Metabolism of 4-chlorophenol by Azotobacter sp. GP1: Structure of the meta cleavage product of 4-chlorocatechol, FEMS Microbiol Lett, 116 (1994) 73-78.
[48] F. Westmeier, H.J. Rehm, Degradation of 4-chlorophenol in municipal wastewater by adsorptiv immobilized Alcaligenes sp. A 7-2, Appl Microbiol Biotechnol, 26 (1987) 78-83.
[49] W.S. El-Sayed, M. Ismaeil, F. El-Beih, Isolation of 4-Chlorophenol-Degrading Bacteria, Bacillus subtilis OS1 and Alcaligenes sp. OS2 from Petroleum Oil-Contaminated Soil and Characterization of its Catabolic Pathway, Aust J Basic Appl Sci 3(2009) 776-789.
[50] H.S. Bae, J.M. Lee, S.T. Lee, Biodegradation of 4-chlorophenol via a hydroquinone pathway by Arthrobacter ureafaciens CPR706, FEMS Microbiol Lett, 145 (1996) 125-129.
[51] K. Nordin, M. Unell, J.K. Jansson, Novel 4-chlorophenol degradation gene cluster and degradation route via hydroxyquinol in Arthrobacter chlorophenolicus A6, Appl Environ Microbiol, 71 (2005) 6538-6544.
[52] L. Padilla, V. Matus, P. Zenteno, B. Gonzalez, Degradation of 2,4,6-trichlorophenol via chlorohydroxyquinol in Ralstonia eutropha JMP134 and JMP222, J Basic Microbiol, 40 (2000) 243-249.
[53] S.D. Copley, Evolution of a metabolic pathway for degradation of a toxic xenobiotic: the patchwork approach, Trends Biochem Sci, 25 (2000) 261-265.
[54] T. Schenk, R. Muller, F. Lingens, Mechanism of enzymatic dehalogenation of pentachlorophenol by Arthrobacter sp. strain ATCC 33790, J Bacteriol, 172 (1990) 7272-7274.
[55] L. Xun, C.S. Orser, Purification and properties of pentachlorophenol hydroxylase, a flavoprotein from Flavobacterium sp. strain ATCC 39723, J Bacteriol, 173 (1991) 4447-4453.
[56] M. Dai, J.B. Rogers, J.R. Warner, S.D. Copley, A previously unrecognized step in pentachlorophenol degradation in Sphingobium chlorophenolicum is catalyzed by tetrachlorobenzoquinone reductase (PcpD), J Bacteriol, 185 (2003) 302-310.

[57] G.V. Reddy, M.H. Gold, Degradation of pentachlorophenol by Phanerochaete chrysosporium: intermediates and reactions involved, Microbiology, 146 (Pt 2) (2000) 405-413.
[58] O. Rubilar, G. Feijoo, C. Diez, T.A. Lu-Chau, M.T. Moreira, J.M. Lema, Biodegradation of Pentachlorophenol in Soil Slurry Cultures by Bjerkandera adusta and Anthracophyllum discolor, Industrial & Engineering Chemistry Research, 46 (2007) 6744-6751.
[59] M.B. Carvalho, S. Tavares, J. Medeiros, O. Nunez, H. Gallart-Ayala, M.C. Leitao, M.T. Galceran, A. Hursthouse, C.S. Pereira, Degradation pathway of pentachlorophenol by Mucor plumbeus involves phase II conjugation and oxidation-reduction reactions, J Hazard Mater, 198 (2011) 133-142.
[60] D.L. McCarthy, A.A. Claude, S.D. Copley, In vivo levels of chlorinated hydroquinones in a pentachlorophenol-degrading bacterium, Appl Environ Microbiol, 63 (1997) 1883-1888.
[61] M. Cai, L. Xun, Organization and regulation of pentachlorophenol-degrading genes in Sphingobium chlorophenolicum ATCC 39723, J Bacteriol, 184 (2002) 4672-4680.
[62] L. Chen, K. Maloney, E. Krol, B. Zhu, J. Yang, Cloning, overexpression, purification, and characterization of the maleylacetate reductase from Sphingobium chlorophenolicum strain ATCC 53874, Curr Microbiol, 58 (2009) 599-603.
[63] M.M. Haggblom, D. Janke, M.S. Salkinoja-Salonen, Hydroxylation and Dechlorination of Tetrachlorohydroquinone by Rhodococcus sp. Strain CP-2 Cell Extracts, Appl Environ Microbiol, 55 (1989) 516-519.
[64] J.H. Apajalahti, M.S. Salkinoja-Salonen, Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced Rhodococcus chlorophenolicus, J Bacteriol, 169 (1987) 5125-5130.
[65] G.V. Reddy, M.D. Gelpke, M.H. Gold, Degradation of 2,4,6-trichlorophenol by Phanerochaete chrysosporium: involvement of reductive dechlorination, J Bacteriol, 180 (1998) 5159-5164.
[66] M.M. Haggblom, L.J. Nohynek, M.S. Salkinoja-Salonen, Degradation and O-methylation of chlorinated phenolic compounds by Rhodococcus and Mycobacterium strains, Appl Environ Microbiol, 54 (1988) 3043-3052.
[67] J.S. Uotila, M.S. Salkinoja-Salonen, J.H. Apajalahti, Dechlorination of pentachlorophenol by membrane bound enzymes of Rhodococcus chlorophenolicus PCP-I, Biodegradation, 2 (1991) 25-31.
[68] J. Dolfing, B.K. Harrison, Gibbs free energy of formation of halogenated aromatic compounds and their potential role as electron acceptors in anaerobic environments, Environ Sci Technol, 26 (1992) 2213-2218.
[69] C. Holliger, G. Wohlfarth, G. Diekert, Reductive dechlorination in the energy metabolism of anaerobic bacteria, FEMS Microbiol Rev, 22 (1998) 383-398.
[70] D.K. Nicholson, S.L. Woods, J.D. Istok, D.C. Peek, Reductive dechlorination of chlorophenols by a pentachlorophenol- acclimated methanogenic consortium, Appl Environ Microbiol, 58 (1992) 2280-2286.
[71] P. Juteau, R. Beaudet, G. McSween, F. Lépine, S. Milot, J.G. Bisaillon, Anaerobic biodegradation of pentachlorophenol by a methanogenic consortium, Appl Microbiol Biotechnol, 44 (1995) 218-224.
[72] B. Bouchard, R. Beaudet, R. Villemur, G. McSween, F. Lepine, J.G. Bisaillon, Isolation and characterization of Desulfitobacterium frappieri sp. nov., an anaerobic bacterium which reductively dechlorinates pentachlorophenol to 3-chlorophenol, Int J Syst Bacteriol, 46 (1996) 1010-1015.
[73] D. Puyol, A.F. Mohedano, J.J. Rodriguez, J.L. Sanz, Effect of 2,4,6-trichlorophenol on the microbial activity of adapted anaerobic granular sludge bioaugmented with Desulfitobacterium strains, N Biotechnol, 29 (2011) 79-89.

[74] M.M. Haggblom, L.Y. Young, Chlorophenol degradation coupled to sulfate reduction, Appl Environ Microbiol, 56 (1990) 3255-3260.
[75] J. Kazumi, M.M. Haggblom, L.Y. Young, Degradation of Monochlorinated and Nonchlorinated Aromatic Compounds under Iron-Reducing Conditions, Appl Environ Microbiol, 61 (1995) 4069-4073.
[76] H.S. Bae, T. Yamagishi, Y. Suwa, Evidence for degradation of 2-chlorophenol by enrichment cultures under denitrifying conditions, Microbiology, 148 (2002) 221-227.
[77] W. Jianlong, N. Horan, E.D. Stentiford, Q. Yi, Anaerobic degradation of 4-chlorophenol under denitrifying conditions with nitrate as the only electron acceptor, Toxicological & Environmental Chemistry, 80 (2001) 209-216.
[78] H.S. Bae, T. Yamagishi, Y. Suwa, Developing and sustaining 3-chlorophenol-degrading populations in up-flow anaerobic column reactors under circum-denitrifying conditions, Appl Microbiol Biotechnol, 59 (2002) 118-124.
[79] H.S. Bae, T. Yamagishi, Y. Suwa, An anaerobic continuous-flow fixed-bed reactor sustaining a 3-chlorobenzoate-degrading denitrifying population utilizing versatile electron donors and acceptors, Chemosphere, 55 (2004) 93-100.
[80] J. Dolfing, Reductive dechlorination of 3-chlorobenzoate is coupled to ATP production and growth in an anaerobic bacterium, strain DCB-1, Arch Microbiol, 153 (1990) 264-266.
[81] W.W. Mohn, K.J. Kennedy, Reductive dehalogenation of chlorophenols by Desulfomonile tiedjei DCB-1, Appl Environ Microbiol, 58 (1992) 1367-1370.
[82] I. Utkin, C. Woese, J. Wiegel, Isolation and characterization of Desulfitobacterium dehalogenans gen. nov., sp. nov., an anaerobic bacterium which reductively dechlorinates chlorophenolic compounds, Int J Syst Bacteriol, 44 (1994) 612-619.
[83] N. Christiansen, B.K. Ahring, Desulfitobacterium hafniense sp. nov., an Anaerobic, Reductively Dechlorinating Bacterium, Int J Syst Bacteriol 46 (1996) 442-448.
[84] R.A. Sanford, J.R. Cole, F.E. Loffler, J.M. Tiedje, Characterization of Desulfitobacterium chlororespirans sp. nov., which grows by coupling the oxidation of lactate to the reductive dechlorination of 3-chloro-4-hydroxybenzoate, Appl Environ Microbiol, 62 (1996) 3800-3808.
[85] B. Sun, J.R. Cole, R.A. Sanford, J.M. Tiedje, Isolation and characterization of Desulfovibrio dechloracetivorans sp. nov., a marine dechlorinating bacterium growing by coupling the oxidation of acetate to the reductive dechlorination of 2-chlorophenol, Appl Environ Microbiol, 66 (2000) 2408-2413.
[86] Q. He, R.A. Sanford, Induction characteristics of reductive dehalogenation in the ortho-halophenol-respiring bacterium, Anaeromyxobacter dehalogenans, Biodegradation, 13 (2002) 307-316.
[87] B.A. van de Pas, H. Smidt, W.R. Hagen, J. van der Oost, G. Schraa, A.J. Stams, W.M. de Vos, Purification and molecular characterization of ortho-chlorophenol reductive dehalogenase, a key enzyme of halorespiration in Desulfitobacterium dehalogenans, J Biol Chem, 274 (1999) 20287-20292.
[88] P. Schmitt, C. Diviès, R. Cardona, Origin of end-products from the co-metabolism of glucose and citrate by Leuconostoc mesenteroides subsp. cremoris, Appl Microbiol Biotechnol, 36 (1992) 679-683.
[89] M. Alexander, Cometabolism., Biodégradation and Bioremediation, Academic Press1994, pp. 177-195.
[90] Q. Wang, Y. Li, J. Li, Y. Wang, C. Wang, P. Wang, Experimental and kinetic study on the cometabolic biodegradation of phenol and 4-chlorophenol by psychrotrophic Pseudomonas putida LY1, Environmental Science and Pollution Research, (2014) 1-9.
[91] S.E. Murialdo, R. Fenoglio, P.M. Haure, J.F. Gonzalez, Degradation of phenol and chlorophenols by mixed and pure cultures, Water SA, 29 (2003) 457-463.

[92] D.S. Shen, X.W. Liu, H.J. Feng, Effect of easily degradable substrate on anaerobic degradation of pentachlorophenol in an upflow anaerobic sludge blanket (UASB) reactor, J Hazard Mater, 119 (2005) 239-243.
[93] E. Sahinkaya, F.B. Dilek, Effect of biogenic substrate concentration on the performance of sequencing batch reactor treating 4-CP and 2,4-DCP mixtures, J Hazard Mater, 128 (2006) 258-264.
[94] M. Tobajas, V.M. Monsalvo, A.F. Mohedano, J.J. Rodriguez, Enhancement of cometabolic biodegradation of 4-chlorophenol induced with phenol and glucose as carbon sources by Comamonas testosteroni, J Environ Manage, 95, Supplement (2012) S116-S121.
[95] K. McAllister, H. Lee, J. Trevors, Microbial degradation of pentachlorophenol, Biodegradation, 7 (1996) 1-40.
[96] O.J. Hao, M.H. Kim, E.A. Seagren, H. Kim, Kinetics of phenol and chlorophenol utilization by Acinetobacter species, Chemosphere, 46 (2002) 797-807.
[97] M.H. Kim, O.J. Hao, Cometabolic degradation of chlorophenols by Acinetobacter species, Water Res, 33 (1999) 562-574.
[98] S.-J. Wang, K.-C. Loh, Biotransformation kinetics of Pseudomonas putida for cometabolism of phenol and 4-chlorophenol in the presence of sodium glutamate, Biodegradation, 12 (2001) 189-199.
[99] S.-J. Wang, K.-C. Loh, New cell growth pattern on mixed substrates and substrate utilization in cometabolic transformation of 4-chlorophenol, Water Res, 34 (2000) 3786-3794.
[100] X. Quan, H. Shi, Y. Zhang, J. Wang, Y. Qian, Biodegradation of 2,4-dichlorophenol and phenol in an airlift inner-loop bioreactor immobilized with Achromobacter sp, Separation and Purification Technology, 34 (2004) 97-103.
[101] C. Chin Wang, C. Mei Lee, C. Jen Lu, M. Shang Chuang, C. Zong Huang, Biodegradation of 2,4,6-trichlorophenol in the presence of primary substrate by immobilized pure culture bacteria, Chemosphere, 41 (2000) 1873-1879.
[102] D. Liu, R.J. Maguire, G. Pacepavicius, B.J. Dutka, Biodegradation of recalcitrant chlorophenols by cometabolism, Environmental Toxicology and Water Quality, 6 (1991) 85-95.
[103] P.R. Senthilnathan, J.J. Ganczarczyk, Adaptation and deadaptation kinetics of activated sludge. 43rd Purdue University Industrial Waste Conference Proceedings, Lewis Publishers Inc.1988.
[104] E. Sahinkaya, F.B. Dilek, Biodegradation of 4-chlorophenol by acclimated and unacclimated activated sludge—Evaluation of biokinetic coefficients, Environ Res, 99 (2005) 243-252.
[105] F.-x. Ye, D.-s. Shen, Acclimation of anaerobic sludge degrading chlorophenols and the biodegradation kinetics during acclimation period, Chemosphere, 54 (2004) 1573-1580.
[106] B.A. Wiggins, S.H. Jones, M. Alexander, Explanations for the acclimation period preceding the mineralization of organic chemicals in aquatic environments, Appl Environ Microbiol, 53 (1987) 791-796.
[107] S. Boyd, D. Shelton, Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge, Appl Environ Microbiol, 47 (1984) 272 - 277.
[108] S.-M. Liu, C.-E. Kuo, T.-B. Hsu, Reductive dechlorination of chlorophenols and pentachlorophenol in anoxic estuarine sediments, Chemosphere, 32 (1996) 1287-1300.
[109] M.A. Sanchez, M. Vasquez, B. Gonzalez, A previously unexposed forest soil microbial community degrades high levels of the pollutant 2,4,6-trichlorophenol, Appl Environ Microbiol, 70 (2004) 7567-7570.
[110] J. Caliz, X. Vila, E. Marti, J. Sierra, J. Nordgren, P.E. Lindgren, L. Baneras, G. Montserrat, The microbiota of an unpolluted calcareous soil faces up chlorophenols:

Evidences of resistant strains with potential for bioremediation, Chemosphere, 83 (2011) 104-116.
[111] M.M. Laine, H. Haario, K.S. Jørgensen, Microbial functional activity during composting of chlorophenol-contaminated sawmill soil, J Microbiol Methods, 30 (1997) 21-32.
[112] Y.-G. Cho, S.-K. Rhee, S.-T. Lee, Influence of environmental parameters on bioremediation of chlorophenol-contaminated soil by indigenous microorganisms, Environ Eng Res 5(2000) 165-173.
[113] M.M. Laine, K.S. Jørgensen, Effective and Safe Composting of Chlorophenol-Contaminated Soil in Pilot Scale, Environ Sci Technol, 31 (1997) 371-378.
[114] A. Sharma, I.S. Thakur, P. Dureja, Enrichment, isolation and characterization of pentachlorophenol degrading bacterium Acinetobacter sp. ISTPCP-3 from effluent discharge site, Biodegradation, 20 (2009) 643-650.
[115] R. Chandra, A. Ghosh, R.K. Jain, S. Singh, Isolation and characterization of two potential pentachlorophenol degrading aerobic bacteria from pulp paper effluent sludge, J Gen Appl Microbiol, 52 (2006) 125-130.
[116] C.F. Yang, C.M. Lee, C.C. Wang, Isolation and physiological characterization of the pentachlorophenol degrading bacterium Sphingomonas chlorophenolica, Chemosphere, 62 (2006) 709-714.
[117] R.F. Al-Thani, D.A.M. Abd-El-Haleem, A.-S. M., Isolation, biochemical and molecular characterization of 2-chlorophenol-degrading Bacillus isolates Afr J Biotechnol 6(2007) 2675-2681.
[118] L. Zhaoxia, D. Cheng, Y. Jinglong, H. Chunmin, Isolation, Identification and Degradation Characterization of p-Chlorophenol Degrading Strain, Education Technology and Training, 2008. and 2008 International Workshop on Geoscience and Remote Sensing. ETT and GRS 2008. International Workshop on, 2008, pp. 293-297.
[119] A.Z. Elkarmi, K.H. Abu-Elteen, A.A. Atta, N.A. Abu-Sbitan, Biodegradation of 2,4-dichlorophenol originating from pharmaceutical industries, Afr J Biotechno, 8 (2009) 2558-2564.
[120] J. Wen, H. Li, J. Bai, Y. Jiang, Biodegradation of 4-Chlorophenol by Candida albicans PDY-07 under Anaerobic Conditions, Chinese Journal of Chemical Engineering, 14 (2006) 790-795.
[121] Y. Jiang, N. Ren, X. Cai, D. Wu, L. Qiao, S. Lin, Biodegradation of Phenol and 4-Chlorophenol by the Mutant Strain CTM 2, Chinese Journal of Chemical Engineering, 16 (2008) 796-800.
[122] S.K. Karn, S.K. Chakrabarty, M.S. Reddy, Pentachlorophenol degradation by Pseudomonas stutzeri CL7 in the secondary sludge of pulp and paper mill, J Environ Sci (China), 22 (2010) 1608-1612.
[123] C.M. Kao, J.K. Liu, Y.L. Chen, C.T. Chai, S.C. Chen, Factors affecting the biodegradation of PCP by Pseudomonas mendocina NSYSU, J Hazard Mater, 124 (2005) 68-73.
[124] S.M. Resnick, P.J. Chapman, Physiological properties and substrate specificity of a pentachlorophenol-degrading Pseudomonas species, Biodegradation, 5 (1994) 47-54.
[125] K.T. Leung, M.B. Cassidy, K.W. Shaw, H. Lee, J.T. Trevors, E.M. Lohmeier-Vogel, H.J. Vogel, Pentachlorophenol biodegradation by Pseudomonas spp. UG25 and UG30, World Journal of Microbiology and Biotechnology, 13 (1997) 305-313.
[126] A. Gallego, V. Gemini, S. Rossi, M.S. Fortunato, E. Planes, C.E. Gómez, S.E. Korol, Detoxification of 2,4,6-trichlorophenol by an indigenous bacterial community, International Biodeterioration & Biodegradation, 63 (2009) 1073-1078.

[127] L. Kharoune, M. Kharoune, J.M. Lebeault, Aerobic degradation of 2,4,6-trichlorophenol by a microbial consortium - selection and characterization of microbial consortium, Appl Microbiol Biotechnol, 59 (2002) 112-117.
[128] C.F. Yang, C.M. Lee, Enrichment, isolation, and characterization of 4-chlorophenol-degrading bacterium Rhizobium sp. 4-CP-20, Biodegradation, 19 (2008) 329-336.
[129] K. Westerberg, A.M. Elvang, E. Stackebrandt, J.K. Jansson, Arthrobacter chlorophenolicus sp. nov., a new species capable of degrading high concentrations of 4-chlorophenol, Int J Syst Evol Microbiol, 50 Pt 6 (2000) 2083-2092.
[130] J.E. Rogers, J. Struijs, D.D. Hale, F. Bryant, Degradation kinetics of chlorinated aromatic compounds in saturated subsurface environments. U.S. Environmental Protection Agency, Environmental Research Laboratory, Athens, GA, (1990).
[131] B.R. Genthner, W.A. Price, P.H. Pritchard, Anaerobic Degradation of Chloroaromatic Compounds in Aquatic Sediments under a Variety of Enrichment Conditions, Appl Environ Microbiol, 55 (1989) 1466-1471.
[132] G.W. Kohring, X.M. Zhang, J. Wiegel, Anaerobic dechlorination of 2,4-dichlorophenol in freshwater sediments in the presence of sulfate, Appl Environ Microbiol, 55 (1989) 2735-2737.
[133] M.M. Häggblom, I.D. Bossert, Halogenated organic compounds - a global perspective, in: M.M. Häggblom, I.D. Bossert (Eds.) Dehalogenation: Microbial Processes and Environmental Applications, Kluwer Academic Publishers, New York, 2003, pp. 3-29.
[134] S. Susarla, Y. Yonezawa, S. Masunaga, Reductive transformations of halogenated aromatics in anaerobic estuarine sediment: kinetics, products and pathways, Water Res, 32 (1998) 639-648.
[135] M.M. Haggblom, M.D. Rivera, L.Y. Young, Influence of alternative electron acceptors on the anaerobic biodegradability of chlorinated phenols and benzoic acids, Appl Environ Microbiol, 59 (1993) 1162-1167.
[136] J. Aguayo, R. Barra, J. Becerra, M. Martínez, Degradation of 2,4,6-tribromophenol and 2,4,6-trichlorophenol by aerobic heterotrophic bacteria present in psychrophilic lakes, World Journal of Microbiology and Biotechnology, 25 (2009) 553-560.
[137] M.K. Mannisto, M.S. Salkinoja-Salonen, J.A. Puhakka, In situ polychlorophenol bioremediation potential of the indigenous bacterial community of boreal groundwater, Water Res, 35 (2001) 2496-2504.
[138] A. Davis, J. Campbell, C. Gilbert, M.V. Ruby, M. Bennett, S. Tobin, Attenuation and Biodegradation of Chlorophenols in Ground Water at a Former Wood Treating Facility, Ground Water, 32 (1994) 248-257.
[139] J. Galíndez-Mayer, J. Ramón-Gallegos, N. Ruiz-Ordaz, C. Juárez-Ramírez, A. Salmerón-Alcocer, H.M. Poggi-Varaldo, Phenol and 4-chlorophenol biodegradation by yeast Candida tropicalis in a fluidized bed reactor, Biochemical Engineering Journal, 38 (2008) 147-157.
[140] E.S. Melin, J.F. Ferguson, J.A. Puhakka, Pentachlorophenol biodegradation kinetics of an oligotrophic fluidized-bed enrichment culture, Appl Microbiol Biotechnol, 47 (1997) 675-682.
[141] S. Eker, F. Kargi, Performance of a hybrid-loop bioreactor system in biological treatment of 2,4,6-tri-chlorophenol containing synthetic wastewater: effects of hydraulic residence time, J Hazard Mater, 144 (2007) 86-92.
[142] S. Eker, F. Kargi, Biological treatment of 2,4,6-trichlorophenol (TCP) containing wastewater in a hybrid bioreactor system with effluent recycle, J Environ Manage, 90 (2009) 692-698.
[143] C. Visvanathan, L.N. Thu, V. Jegatheesan, J. Anotai, Biodegradation of pentachlorophenol in a membrane bioreactor, Desalination, 183 (2005) 455-464.

[144] B.C. Alleman, B.E. Logan, R.L. Gilbertson, Degradation of pentachlorophenol by fixed films of white rot fungi in rotating tube bioreactors, Water Res, 29 (1995) 61-67.
[145] A. Leontievsky, N. Myasoedova, L. Golovleva, M. Sedarati, C. Evans, Adaptation of the white-rot basidiomycete Panus tigrinus for transformation of high concentrations of chlorophenols, Appl Microbiol Biotechnol, 59 (2002) 599-604.
[146] M.R. Sedarati, T. Keshavarz, A.A. Leontievsky, C.S. Evans, Transformation of high concentrations of chlorophenols by the white-rot basidiomycete Trametes versicolor immobilized on nylon mesh, Electron J Biotechnol 6(2003) 104-114.
[147] N. Pal, G. Lewandowski, P.M. Armenante, Process optimization and modeling of trichlorophenol degradation by Phanerochaete chrysosporium, Biotechnol Bioeng, 46 (1995) 599-609.
[148] F.T. Saia, M.H. Damianovic, E.B. Cattony, G. Brucha, E. Foresti, R.F. Vazoller, Anaerobic biodegradation of pentachlorophenol in a fixed-film reactor inoculated with polluted sediment from Santos-Sao Vicente Estuary, Brazil, Appl Microbiol Biotechnol, 75 (2007) 665-672.
[149] E.I. Atuanya, T. Chakrabarti, Biotreatability and kinetics of UASB reactor to mixtures of chlorophenol pollutants, Environ Monit Assess, 83 (2003) 283-294.
[150] P.S. Majumder, S.K. Gupta, Degradation of 4-chlorophenol in UASB reactor under methanogenic conditions, Bioresour Technol, 99 (2008) 4169-4177.
[151] D.S. Shen, X.W. Liu, Y.H. He, Studies on adsorption, desorption and biodegradation of pentachlorophenol by the anaerobic granular sludge in an upflow anaerobic sludge blanket (UASB) reactor, J Hazard Mater, 125 (2005) 231-236.
[152] F.X. Ye, Y. Li, Biosorption and biodegradation of pentachlorophenol (PCP) in an upflow anaerobic sludge blanket (UASB) reactor, Biodegradation, 18 (2007) 617-624.
[153] B. Tartakovsky, M. Levesque, R. Dumortier, R. Beaudet, S.R. Guiot, Biodegradation of pentachlorophenol in a continuous anaerobic reactor augmented with Desulfitobacterium frappieri PCP-1, Appl Environ Microbiol, 65 (1999) 4357-4362.
[154] E.A. Baraldi, M.H. Damianovic, G.P. Manfio, E. Foresti, R.F. Vazoller, Performance of a horizontal-flow anaerobic immobilized biomass (HAIB) reactor and dynamics of the microbial community during degradation of pentachlorophenol (PCP), Anaerobe, 14 (2008) 268-274.
[155] M.H. Damianovic, E.M. Moraes, M. Zaiat, E. Foresti, Pentachlorophenol (PCP) dechlorination in horizontal-flow anaerobic immobilized biomass (HAIB) reactors, Bioresour Technol, 100 (2009) 4361-4367.
[156] V.S. Magar, H.D. Stensel, J.A. Puhakka, J.F. Ferguson, Sequential anaerobic dechlorination of pentachlorophenol: competitive inhibition effects and a kinetic model, Environ Sci Techno 33 (1999) 1604-1611.
[157] H.M. Poggi-Varaldo, J.D. Barcenas-Torres, C.U. Moreno-Medina, J. Garcia-Mena, C. Garibay-Orijel, E. Rios-Leal, N. Rinderknecht-Seijas, Influence of discontinuing feeding degradable cosubstrate on the performance of a fluidized bed bioreactor treating a mixture of trichlorophenol and phenol, J Environ Manage, 113 (2012) 527-537.
[158] J. Lopez, V.M. Monsalvo, D. Puyol, A.F. Mohedano, J.J. Rodriguez, Low-temperature anaerobic treatment of low-strength pentachlorophenol-bearing wastewater, Bioresour Technol, 140 (2013) 349-356.
[159] Z. Li, Y. Inoue, S. Yang, N. Yoshida, A. Katayama, Mass balance and kinetic analysis of anaerobic microbial dechlorination of pentachlorophenol in a continuous flow column, J Biosci Bioeng, 110 (2010) 326-332.
[160] M. Bajaj, C. Gallert, J. Winter, Anaerobic biodegradation of high strength 2-chlorophenol-containing synthetic wastewater in a fixed bed reactor, Chemosphere, 73 (2008) 705-710.

[161] P.M. Armenante, D. Kafkewitz, G.A. Lewandowski, C.-J. Jou, Anaerobic–aerobic treatment of halogenated phenolic compounds, Water Res, 33 (1999) 681-692.
[162] P.S. Majumder, S.K. Gupta, Removal of chlorophenols in sequential anaerobic-aerobic reactors, Bioresour Technol, 98 (2007) 118-129.
[163] S.K. Bhattacharya, Q. Yuan, P. Jin, Removal of pentachlorophenol from wastewater by combined anaerobic-aerobic treatment, J Hazard Mater, 49 (1996) 143-154.
[164] G.J. Wilson, A.P. Khodadoust, M.T. Suidan, R.C. Brenner, Anaerobic/aerobic biodegradation of pentachlorophenol using GAC fluidized bed reactors: Optimization of the empty bed contact time, Water Science and Technology, 36 (1997) 107-115.
[165] T. Al-Khalid, M.H. El-Naas, Aerobic Biodegradation of Phenols: A Comprehensive Review, Critical Reviews in Environmental Science and Technology, 42 (2012) 1631-1690.
[166] V.H. Edwards, The influence of high substrate concentrations on microbial kinetics, Biotechnol Bioeng, 12 (1970) 679-712.
[167] J.F. Andrews, A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates, Biotechnol Bioeng, 10 (1968) 707-723.
[168] G.M. Klecka, W.J. Maier, Kinetics of microbial growth on pentachlorophenol, Appl Environ Microbiol, 49 (1985) 46-53.
[169] N.K. Sahoo, K. Pakshirajan, P.K. Ghosh, A. Ghosh, Biodegradation of 4-chlorophenol by Arthrobacter chlorophenolicus A6: effect of culture conditions and degradation kinetics, Biodegradation, 22 (2011) 275-286.
[170] B. Basak, B. Bhunia, S. Dutta, A. Dey, Enhanced biodegradation of 4-chlorophenol by Candida tropicalis PHB5 via optimization of physicochemical parameters using Taguchi orthogonal array approach, International Biodeterioration & Biodegradation, 78 (2013) 17-23.
[171] J. Balfanz, H.-J. Rehm, Biodegradation of 4-chlorophenol by adsorptive immobilized Alcaligenes sp. A 7-2 in soil, Appl Microbiol Biotechnol, 35 (1991) 662-668.
[172] G. Annadurai, R.-S. Juang, D.-J. Lee, Microbiological degradation of phenol using mixed liquors of Pseudomonas putida and activated sludge, Waste Management, 22 (2002) 703-710.
[173] G. Annadurai, L.Y. Ling, J.-F. Lee, Statistical optimization of medium components and growth conditions by response surface methodology to enhance phenol degradation by Pseudomonas putida, J Hazard Mater, 151 (2008) 171-178.
[174] D.C. Montgomery, Design and Analysis of Experiments, 3rd ed., Wiley, New York, 1991.
[175] S.S. Bhattacharya, R. Banerjee, Laccase mediated biodegradation of 2,4-dichlorophenol using response surface methodology, Chemosphere, 73 (2008) 81-85.
[176] S.D. Shinde, S.S. Lele, Statistical media optimization for lutein production from microalgae Auxenochlorella protothecoides SAG 211-7A, International Journal of Advanced Biotechnology and Research, 1 (2010) 104-114.
[177] B. Qi, X. Chen, F. Shen, Y. Su, Y. Wan, Optimization of Enzymatic Hydrolysis of Wheat Straw Pretreated by Alkaline Peroxide Using Response Surface Methodology, Industrial & Engineering Chemistry Research, 48 (2009) 7346-7353.
[178] M.Z. Khan, P.K. Mondal, S. Sabir, Bioremediation of 2-chlorophenol containing wastewater by aerobic granules-kinetics and toxicity, J Hazard Mater, 190 (2011) 222-228.
[179] M. Goswami, N. Shivaraman, R.P. Singh, Kinetics of chlorophenol degradation by benzoate-induced culture of Rhodococcus erythropolis M1, World Journal of Microbiology and Biotechnology, 18 (2002) 779-783.
[180] A. Chiavola, R. Baciocchi, F. Barducci, 3-Chlorophenol Biodegradation in a Sequencing Batch Reactor: Kinetic Study and Effect of the Filling Time, Water, Air, & Soil Pollution, 212 (2010) 219-229.

[181] E. Sahinkaya, F.B. Dilek, Effect of feeding time on the performance of a sequencing batch reactor treating a mixture of 4-CP and 2,4-DCP, J Environ Manage, 83 (2007) 427-436.
[182] I. Moreno-Andrade, G. Buitrón, Comparison of the Performance of Membrane and Conventional Sequencing Batch Reactors Degrading 4-Chlorophenol, Water, Air, & Soil Pollution, 223 (2012) 2083-2091.
[183] J.-y. Ma, X.-c. Quan, Z.-f. Yang, A.-j. Li, Biodegradation of a mixture of 2,4-dichlorophenoxyacetic acid and multiple chlorophenols by aerobic granules cultivated through plasmid pJP4 mediated bioaugmentation, Chemical Engineering Journal, 181–182 (2012) 144-151.
[184] F. Kargi, S. Eker, Kinetics of 2,4-dichlorophenol degradation by Pseudomonas putida CP1 in batch culture, International Biodeterioration & Biodegradation, 55 (2005) 25-28.
[185] Y. Gu, R.A. Korus, Kinetics of pentachlorophenol degradation by a Flavobacterium species, Appl Microbiol Biotechnol, 43 (1995) 374-378.
[186] M. Rutgers, A.M. Breure, J.G. van Andel, W.A. Duetz, Growth yield coefficients of Sphingomonas sp. strain P5 on various chlorophenols in chemostat culture, Appl Microbiol Biotechnol, 48 (1997) 656-661.
[187] M.Y. Pamukoglu, F. Kargi, Biodegradation kinetics of 2,4,6-trichlorophenol by Rhodococcus rhodochrous in batch culture, Enzyme and Microbial Technology, 43 (2008) 43-47.
[188] E.S. Melin, K.T. Jarvinen, J.A. Puhakka, effects of temperature on chlorophenol biodegradation kinetics in fluidized-bed reactors with different biomass carriers, Water Res, 32 (1998) 81-90.
[189] G.J. Stanlake, R.K. Finn, Isolation and characterization of a pentachlorophenol-degrading bacterium, Appl Environ Microbiol, 44 (1982) 1421-1427.
[190] M. Männistö, M. Tiirola, J. Puhakka, Degradation of 2,3,4,6-tetrachlorophenol at low temperature and low dioxygen concentrations by phylogenetically different groundwater and bioreactor bacteria, Biodegradation, 12 (2001) 291-301.
[191] B.N. Jacobsen, E. Arvin, Biodegradation kinetics and fate modelling of pentachlorophenol in bioaugmented activated sludge reactors, Water Res, 30 (1996) 1184-1194.
[192] R. Edgehill, R. Finn, Isolation, characterization and growth kinetics of bacteria metabolizing pentachlorophenol, European journal of applied microbiology and biotechnology, 16 (1982) 179-184.
[193] S.K. Banedi, R.K. Bajpail, Cometabolism of pentachlorophenol by microbial species, J Hazard Mater, 39 (1994) 19-31.
[194] Z.C. Hu, R.A. Korus, W.E. Levinson, R.L. Crawford, Adsorption and biodegradation of pentachlorophenol by polyurethane-immobilized flavobacterium, Environ Sci Technol, 28 (1994) 491-496.
[195] M. Rutgers, D.D. Gooch, A.M. Breure, J.G. Van Andel, Assessment of inhibition kinetics of the growth of strain P5 on pentachlorophenol under steady-state conditions in a nutristat, Arch Microbiol, 165 (1996) 194-200.
[196] G. Dietrich, J. Winter, Anaerobic degradation of chlorophenol by an enrichment culture, Appl Microbiol Biotechnol, 34 (1990) 253-258.
[197] J.R. Cole, A.L. Cascarelli, W.W. Mohn, J.M. Tiedje, Isolation and characterization of a novel bacterium growing via reductive dehalogenation of 2-chlorophenol, Appl Environ Microbiol, 60 (1994) 3536-3542.
[198] J. Dolfing, J.M. Tiedje, Growth yield increase linked to reductive dechlorination in a defined 3-chlorobenzoate degrading methanogenic coculture, Arch Microbiol, 149 (1987) 102-105.

[199] H. Hendriksen, B. Ahring, Metabolism and kinetics of pentachlorophenol transformation in anaerobic granular sludge, Appl Microbiol Biotechnol, 37 (1992) 662-666.
[200] X.W. Liu, R. He, D.S. Shen, Studies on the toxic effects of pentachlorophenol on the biological activity of anaerobic granular sludge, J Environ Manage, 88 (2008) 939-946.
[201] S.R. Guiot, B. Tartakovsky, M. Lanthier, M.J. Levesque, M.F. Manuel, R. Beaudet, C.W. Greer, R. Villemur, Strategies for augmenting the pentachlorophenol degradation potential of UASB anaerobic granules, Water Sci Technol, 45 (2002) 35-41.
[202] F.-X. Ye, D.-S. Shen, X.-S. Feng, Anaerobic granule development for removal of pentachlorophenol in an upflow anaerobic sludge blanket (UASB) reactor, Process Biochemistry, 39 (2004) 1249-1256.
[203] J. Monod, The Growth of Bacterial Cultures, Annu Rev Microbiol, 3 (1949) 371-394.
[204] T. Yano, T. Nakahara, S. Kamiyama, K. Yamada, Kinetic Studies on Microbial Activities in Concentrated Solutions, Agricultural and Biological Chemistry, 30 (1966) 42-48.
[205] R.B. Fisher, Enzyme And Metabolic Inhibitors, by J. Leyden Webb. New York and London: Academic Press. Vol.I. Pp.951. 1963. £9 6s. Vol.II. 1966. Pp.xx+1237. 272s. Vol.III. 1966. Pp.xx+1028. 256s, Quarterly Journal of Experimental Physiology and Cognate Medical Sciences, 54 (1969) 103-104.
[206] S. Aiba, M. Shoda, M. Nagatani, Kinetics of product inhibition in alcohol fermentation, Biotechnol Bioeng, 10 (1968) 845-864.
[207] M. Ziagova, M. Liakopoulou-Kyriakides, Kinetics of 2,4-dichlorophenol and 4-Cl-m-cresol degradation by Pseudomonas sp. cultures in the presence of glucose, Chemosphere, 68 (2007) 921-927.
[208] M. Goswami, N. Shivaraman, R. Singh, Kinetics of chlorophenol degradation by benzoate-induced culture of Rhodococcus erythropolis M1, World J Microbiol Biotechnol, 18 (2002) 779 - 783.
[209] R.K. Freier, Wasseranalyse, 2nd ed., Walter de Gruyter, Berlin, 1974.
[210] EPA, Method 9251- Chloride - Colorimetric, Auto Ferricyanide AAII. US Environmental Protection Agency, SW-846 ch5., (1986).
[211] L.E. Arnow, colorimetric determination of the components of 3,4-dihydroxyphenylalaninetyrosine mixtures, Journal of Biological Chemistry, 118 (1937) 531-537.
[212] R.E. Buchanan, N.E. Gibbons, Bergey's Manual of Determinative Bacteriology, 8th ed., The Williams and Wilkins Company, Baltimore, Maryland, 1974.
[213] A. Kapley, K. Lampel, H.J. Purohit, Rapid Detection of Salmonella in Water Samples by Multiplex Polymerase Chain Reaction, Water Environment Research, 73 (2001) 461-465.
[214] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods, Mol Biol Evol, 28 (2011) 2731-2739.
[215] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol Biol Evol, 4 (1987) 406-425.
[216] M.C. Tomei, M.C. Annesini, S. Bussoletti, 4-nitrophenol biodegradation in a sequencing batch reactor: kinetic study and effect of filling time, Water Res, 38 (2004) 375-384.
[217] J.B.S. Haldane, Enzymes, MIT Press, Cambridge,MA, 1965.
[218] Z.I. Finkel'shtein, B.P. Baskunov, E.L. Golovlev, O.V. Moiseeva, J. Vervoort, I. Rietjens, L.A. Golovleva, Dependence of the conversion of chlorophenols by rhodococci on the number and position of chlorine atoms in the aromatic ring, Microbiology, 69 (2000) 40-47.

[219] L. Huang, L. Gan, Q. Zhao, B.E. Logan, H. Lu, G. Chen, Degradation of pentachlorophenol with the presence of fermentable and non-fermentable co-substrates in a microbial fuel cell, Bioresour Technol, 102 (2011) 8762-8768.
[220] F. Kargi, S. Eker, Toxicity and batch biodegradation kinetics of 2,4 dichlorophenol by pure Pseudomonas putida culture, Enzyme and Microbial Technology, 35 (2004) 424-428.
[221] C.C. Wang, C.M. Lee, C.H. Kuan, Removal of 2,4-dichlorophenol by suspended and immobilized Bacillus insolitus, Chemosphere, 41 (2000) 447-452.
[222] A. Papazi, K. Kotzabasis, "Rational" Management of Dichlorophenols Biodegradation by the Microalga Scenedesmus obliquus, PLoS One, 8 (2013) e61682.
[223] G. Baggi, V. Andreoni, S. Bernasconi, L. Cavalca, M. Zangrossi, Co-metabolic degradation of mixtures of monochlorophenols by phenol-degrading microorganisms, Annals of Microbiology, 52 (2002) 133-143.
[224] A. Papazi, K. Kotzabasis, "Rational" management of dichlorophenols biodegradation by the microalga *Scenedesmus obliquus*, PloS one, 8 (2013) e61682.
[225] D.C. Yee, T.K. Wood, 2,4-Dichlorophenol Degradation Using Streptomyces viridosporus T7A Lignin Peroxidase, Biotechnol Prog, 13 (1997) 53-59.
[226] A.M. Farag, H.M. Abd-Elnaby, Degradation of Phenol by A New-Degradable Marine Halophilic Fungus Fennellia Flavipes Isolated From Mangrove Sediments, Life Science Journal, 11 (2014) 836-845.
[227] O. Rubilar, M.C. Diez, L. Gianfreda, Transformation of Chlorinated Phenolic Compounds by White Rot Fungi, Critical Reviews in Environmental Science and Technology, 38 (2008) 227-268.
[228] WHO, Environmental Health Criteria 93: Chlorophenols other than pentachlorophenol, International Programme on Chemical Safety, WHO, Geneva, Switzerland, (1989).
[229] S.A. Boyd, D.R. Shelton, Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge, Appl Environ Microbiol, 47 (1984) 272-277.
[230] R.K. Jain, J.H. Dreisbach, J.C. Spain, Biodegradation of p-nitrophenol via 1,2,4-benzenetriol by an Arthrobacter sp, Appl Environ Microbiol, 60 (1994) 3030-3032.
[231] M. Dilaver, F. Kargi, 2,4-Dichlorophenol (DCP) containing wastewater treatment using a hybrid-loop bioreactor, Bioresour Technol, 100 (2009) 1459-1462.
[232] G. Swaminathan, T.K. Ramanujam, Effect of substrate concentration on biodegradation of 2,4-dichlorophenol using modified rotating biological contactors, Bioprocess Engineering, 21 (1999) 169-173.
[233] A. Uysal, A. Türkman, Effect of biosurfactant on 2,4-dichlorophenol biodegradation in an activated sludge bioreactor, Process Biochemistry, 40 (2005) 2745-2749.
[234] F. Kargi, I. Konya, Para-chlorophenol containing synthetic wastewater treatment in an activated sludge unit: Effects of hydraulic residence time, J Environ Manage, 84 (2007) 20-26.
[235] T.M. Louie, C.M. Webster, L. Xun, Genetic and Biochemical Characterization of a 2,4,6-Trichlorophenol Degradation Pathway in Ralstonia eutropha JMP134, Journal of Bacteriology, 184 (2002) 3492–3500.

APPENDIX – I

Mineral salt medium (Modified DSMZ 465):

Ingredients	**g/L**
$Na_2HPO_4 \cdot 2H_2O$	3.5
KH_2PO_4	1
$(NH_4)_2SO_4$	0.5
$MgCl_2 \cdot 6H_2O$	0.1
$Ca(NO_3)_2 \cdot 4H_2O$	0.05
Trace element solution - A	1 mL
Trace element Solution - A	**g/L**
EDTA	0.5
$FeSO_4 \cdot 7H_2O$	0.2
Trace element solution - B	100 mL
D/W	900mL
Trace element solution - B	**g/L**
$CuCl_2 \cdot 2H_2O$	0.01
$ZnSO_4 \cdot 7H_2O$	0.1
$MnCl_2 \cdot 4H_2O$	0.03
$CoCl_2 \cdot 6H_2O$	0.2
H3BO3	0.3
$NiCl_2 \cdot 6H_2O$	0.02
$Na_2MoO_4 \cdot 2H_2O$	0.03
D/W	1000 mL

Nutrient broth:

Ingredients	**g/L**
Peptone	5
Yeast extract	1.5
Beef extract	1.5
NaCL	5
D/W	1000 mL

Preparation of glycerol stock:

1. The bacterial culture was inoculated 24 h for preparation of the glycerol stock.
2. 700µL of the grown culture was add to 300 µL of autoclaved glycerol and mixed properly and was immediately transferred to ice.
3. It was stored at -20°C for future use.

Phosphate buffer:

Inorganic salt	**mg/L**
Disodium hydrogen phosphate, anhydrous	726
Potassium dihydrogen phosphate	210
Sodium chloride	9000
D/W	1000 mL

SIM medium:

Ingredients	**g/L**
Agar	3
Ammonium ferric citrate	0.2
Casein peptone	20
Meat peptone	6.6
Sodium thiosulfate	0.2
Final pH	7.3±0.2

Simmons Citrate agar:

Ingredients	**g/L**
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0

Final pH (at 25°C)	6.8±0.2

Stuart Urea broth:

Ingredients	**g/L**
Yeast extract	0.1
Dipotassium hydrogen phosphate	9.5
Potassium dihydrogen phosphate	9.1
Urea	20
Phenol red	0.01
pH (at 25 °C)	6.8±0.2

Nitrate broth:

Ingredients	**g/L**
Peptone	5
Meat extract	3
Potassium nitrate	1
Sodium chloride	30
pH (at 25 °C)	7.0±0.2

Starch agar:

Ingredients	**g/L**
Meat extract	3
Peptone	5
Starch soluble	2
Agar	15
pH (at 25 °C)	7.2±0.1

MR-VP medium:

Ingredients	**g/L**
Buffered Peptone	7
Dextrose	5
Dipotassium phosphate	5
pH (at 25 °C)	6.9±0.2

Skim milk agar:

Ingredients	g/L
Skim milk powder	28
Casein enzymic hydrolysate	5
Yeast extract	2.5
Dextrose	1
Agar	15
pH (at 25 °C)	7.0±0.2

Gelatin agar:

Ingredients	g/L
Gelatin	30
Casein enzymic hydrolysate	10
Sodium chloride	10
Agar	15
pH (at 25 °C)	7.2±0.2

PAPER PUBLICATIONS

- **B. P. Patel,** A. Kumar.Biodegradation of 2,4-dichlorophenol by *Bacillus endophyticus* strain: Optimization of experimental parameters using response surface methodology and kinetic study. Desalination and Water Treatment (Accepted).
- **B. P. Patel,** A. Kumar. Optimization Study for Maximizing 2,4-Dichlorophenol Degradation by *Kocuria rhizophila* Strain Using Response Surface Methodology and Kinetic Study. Journal of Desalination and Water Treatment (Accepted).
- **B. P. Patel,** A. Kumar. Distribution, Toxicity and Biotransformation of Chlorophenols in Natural and Engineered System – A Review. Journal of Reviews in Environmental Science and Biotechnology (Revision submitted).
- **B. P. Patel,** A. Kumar. Biodegradation and co-metabolism of monochlorophenols and 2,4-dichlorophenol by microbial consortium. Journal of CLEAN – Soil, Air, Water (Revision submitted).
- **B. P. Patel,** A. Kumar. Biodegradation of 4-chlorophenol in airlift inner loop bioreactor with mixed consortium: effect of HRT, loading rate and biogenic substrate. Journal of 3 Biotech (Under Revision).
- **B. P. Patel,** A. Kumar. Biodegradation of 2,4-dichlorophenol in Packed Bed Biofilm Reactor: Effect of Hydraulic Retention Time, Biogenic Substrate and Loading Rate. Journal of Water Environment Research (Under Revision).
- **B. P. Patel,** A. Kumar. Biodegradation of monochlorophenols and their cometabolism with 2,4-dichlorophenol by defined microbial consortium. Journal of environmental Processes (Under Review).
- **Under communication: 4**

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M.Tech. in Biotechnology — June 2011
First class - 8.65 CGPA

V. V. P. Engineering College — Saurashtra University
B.E. in Biotechnology — June 2009
First class - 61.53%

Research Experience

PhD scholar, NIT Rourkela — 2011-2015
Microbial Degradation of Chlorophenols in Batch and Continuous Bioreactors: Kinetic Study and Optimization of Process Parameters

- Isolated and acclimated and characterized the potent bacterial strains from industrial wastewater and soil.
- Biodegradation, optimization, cometabolism and kinetic study of chlorophenols by pure and mixed culture evaluated.
- Fabricated three customized bioreactors: two packed bed biofilm reactor (PBBR) with 0.45 L and 0.7 L capacity and an Air lift inner loop reactor (ALR) with 12 L capacity to study the effect of hydraulic retention time, substrate loading rate, and effect of biogenic substrate on chlorophenols removal by pure and mixed culture.

MTech research project, NIT Rourkela — 2010-2011
Anaerobic Digestion of kitchen waste and to check the effect of different parameters

- The main objective of the project is to check effect of different parameters (pH, Total solid concentration, Temp., etc.) on biogas production. It included working on a different scale digesters /bioreactors from low cost customized 1 liter digesters to pilot

plant scale level of 20 liter digesters operated under batch and semi continuous mode.

Industrial training in Quality Control and Microbiology Department
Lincoln Pharmaceuticals Ltd., Kalol for Four weeks

Industrial training in R&D Laboratory Plant Tissue Culture & Bio-fertilizer
IFFCO, Kalol for Ten weeks

Current Occupation

- **Research Associate**
  Value Addition Research Development Department
  National Innovation Foundation (An autonomous body of Department of Science and Technology, GOI)